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Potential of resveratrol and genistein to influence marine and freshwater fish fatty acid metabolism and performance

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Experience is what you get when you didn't get what you wanted.

- Randy Pausch, The Last Lecture -

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List of Abbreviations

AA	Amino acid
acox 1	Acyl-CoA oxidase 1
ADC	Apparent digestibility coefficient
ALA	α-linolenic acid (18:3n-3)
AMPK	Adenosine monophosphate (AMP)-activated protein kinase
ANF	Anti-nutritional factor
β-actin	Beta-actin
BCA	Bicinchoninic acid
CEL	Carboxyl ester lipase
CM	Circular muscle
CPT1	Carnitine palmitoyl transferase 1
∆6-D	∆6-desaturase
∆5-D	∆5-desaturase
DFI	Daily feed intake
DHA	Docosahexaenoic acid, Docosahexaensäure (22:6n-3)
DM	Dry matter
DPA	Docosapentaenoic acid (22:5n-3)
EAA	Essential amino acid
ECH	Enoyl-CoA hydratase
EF1α	Elongation factor 1 α
ELISA	Enzyme-linked immunosorbent assay
EP	Mucosal epithelium
EPA	Eicosapentaenoic acid, Eicosapentaensäure (20:5n-3)
FA	Fatty acid
FAME	Fatty acid methyl ester
FBW	Final body weight
FCF	Fulton condition factor
FCR	Feed conversion ratio
g	goblet cell
G	Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-(4H)-benzopyran-4-one)
GC-FID	Gas chromatograph with flame ionization detector
HE	Hematoxylin and eosin
HK	Hematocrit

HRP	Horseradish peroxidase
HSI	Hepatosomatic index
HUFA	Highly unsaturated fatty acids (in this thesis: EPA, DPA, DHA, and arachidonic acid)
IBW	Initial body weight
IF	Internal folding (also: annulo-spiral septum)
LA	Linoleic acid (18:2n-6)
LC-PUFA	Long chain polyunsaturated fatty acid
LM	Longitudinal muscle
LP	Lamina propria
ME	Muscularis externa
MF	Mucosal fold
MUFA	Mono-unsaturated fatty acid
n-3, n-6, n-9	Omega-3, omega-6, omega-9
OM	Original matter
PER	Protein efficiency ratio
PPARα	Peroxisome proliferator-activated receptor α
PPV	Protein productive value
PUFA	Polyunsaturated fatty acid
qRT-PCR	Quantitative real-time reverse transcription polymerase chain reaction
RAS	Recirculating aquaculture system
RSE	Residual standard error
RV	Resveratrol (*trans*-3,4',5-trihydroxy stilbene)
SC	Stratum compactum
SFA	Saturated fatty acid
SG	Stratum granulosum
SGR	Specific growth rate
SM	Submucosa
SREBP-1	Sterol regulatory element binding protein 1
TiO_2	Titanium dioxide
VIE	Visible implant elastomer

GENERAL INTRODUCTION

Human nutrition and aquaculture

The global population is continuously growing and is expected to reach more than eight billion people by 2025 (United Nations Department of Economic and Social Affairs, 2017). Already nowadays, 462 million people worldwide suffer from undernutrition whereas 1.9 billion people face the problem of overnutrition and obesity (NCD Risk Factor Collaboration, 2016; WHO, 2017). There is increasing need to supply the world population with healthy and high-quality foods, stressing the importance of agri- and aquaculture. Especially fisheries and aquaculture are regarded as important components in reducing hunger and malnutrition in poor countries and promoting health in countries facing overnutrition.

The importance of fish for human nutrition is not just the protein quality and the considerable amounts of essential vitamins and minerals, but especially the lipid quality (FAO, 2016). The lipids and fatty acids present in fish make it a unique and qualitatively superior food in contrast to other terrestrial animal and plant food sources. Fish and seafood are especially rich in omega-3 (n-3) highly unsaturated fatty acids (HUFA) that have a carbon chain length of ≥C20 (FAO, 2014, 2010; Sargent et al., 2002; WHO, 2003). The n-3 HUFAs eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), amongst others, play important and unique roles in human nutrition. HUFAs play a fundamental role in the neuronal and visual development besides other physiological, biochemical, and cellular functions (Calder, 2015, 2012; ISSFAL, 2004; Molendi-Coste et al., 2011).

Many health problems of the western-oriented countries facing overnutrition are associated with excessive intake of terrestrial animal fats and plant oils containing high levels of omega-6 (n-6) and low levels of n-3 fatty acids (Molendi-Coste et al., 2011; Simopoulos, 2006). The food producing sectors relying on terrestrial resources are dominated by the n-6 fatty acid linoleic acid (18:2n-6), among other fatty acids as palmitic acid (16:0) and oleic acid (18:1n-9) (Colombo et al., 2017; Gunstone, 2011; Hixson et al., 2015; Sargent et al., 2002). Some n-3 polyunsaturated fatty acids (PUFAs) with chain lengths below C20 are present in terrestrial plants. The oil crops soybean, rapeseed or canola, and linseed contain the n-3 fatty acid α-linolenic acid (ALA, 18:3n-3) (Gunstone, 2011). A balanced diet should aim at an n-6/n-3 ratio of 1/1 but instead, the western-type diet has an n-6/n-3 ratio of up to 20-25/1 (Simopoulos, 2011). Thus, an increased consumption of fish and fish products that contain considerably higher amounts of

n-3 HUFAs is emphasized by the FAO, ISSFAL and WHO, amongst others (FAO, 2014; ISSFAL, 2004; WHO, 2003). Since natural supplies from capture fisheries are becoming increasingly restricted, the aquaculture based production under controlled conditions needs to counterbalance the rising demand for fish and seafood.

Lipids and fatty acids in fish nutrition

In order to produce fish of a high quality and rich in n-3 HUFAs the lipid sources in the artificial aquafeeds play a crucial role. To better understand the choice of lipid source for fish nutrition it is indispensable to highlight the function of lipids in fish, as well as the natural dietary lipid sources and their quality. Lipids and fatty acids play a major role in fish nutrition and are required for vital physiological functions same as in humans and other animals. Fish depend on dietary lipids and fatty acids for their energy metabolism, growth, and membrane structure and functioning (Sargent et al., 2002). The HUFAs and PUFAs are of importance for the ontogenesis, survival, pigmentation, stress and disease resistance, as well as in the development and functionality of the brain, vision, and nervous system (Tocher, 2003; Vagner and Santigosa, 2011). The quality and amount of lipids (composition of fatty acids and HUFAs) in fish depend largely on the species, developmental stage, environmental factors like temperature, and the dietary lipid quality (Schulz et al., 2008; Skonberg et al., 1994; Vagner and Santigosa, 2011). The requirement for PUFAs and HUFAs by each fish species are the result of evolutionary adaptations to a certain environment and the capability of the species to synthesize these fatty acids. A differentiation in fish of the freshwater environment with a so-called freshwater lifestyle and fish of the marine environment with a marine lifestyle is practical from a nutritional point of view.

Freshwater fish

The freshwater environment differs from the marine environment with regard to the distribution of EPA and DHA. Though some freshwater microalgae contain EPA and DHA, the predominant fatty acids are the shorter chained n-3 and n-6 fatty acids ALA and linoleic acid (18:2n-6), respectively (Hixson et al., 2015). Insect larvae and zooplankton feeding on the microalgae can have substantial amounts of EPA and the n-6 HUFA arachidonic acid (20:4n-6), but usually lack DHA (Brett and Müller-Navarra, 1997). Thus, the base of the freshwater food chain contains low amounts of n-3 HUFAs and is dominated by C18 fatty acids. Freshwater fish need to endogenously synthesize EPA and DHA in order to meet their requirement for n-3 HUFAs. Almost all freshwater fish are capable of converting the precursor C18 ALA to the long chain EPA and DHA *via* the *in vivo* hepatic fatty acid synthesis chain. Few exceptions are strictly piscivorous fish like

pike since their prey organisms supply them with sufficient amounts of EPA and DHA. The majority of freshwater fish of economic importance thus have low dietary requirement for n-3 HUFAs and a greater requirement for ALA and linoleic acid (NRC, 2011; Sargent et al., 2002).

Marine fish

The marine environment is dominated by HUFAs like EPA and DHA, which are *de novo* synthesized by marine microalgae (Colombo et al., 2017). These HUFAs are enriched along the natural food chain: HUFA-rich microalgae in the phytoplankton are ingested by zooplankton which are eaten by planktivorous fish, which are eventually the prey of carnivorous/piscivorous fish. Thus, marine fish are supplied *via* their diet with sufficient amounts of n-3 HUFAs. This all-time supply of marine fish with EPA and DHA has led to cellular, molecular, and transcriptional adaptations of the fatty acid metabolism and synthesis through evolutionary processes (Sargent et al., 2002; Tocher, 2003; Yamada et al., 1980). In marine fish, the *in vivo* hepatic fatty acid synthesis converting the precursor ALA to EPA and DHA only proceeds partly (Tocher, 2003). Though the genetic capacity is present, some genes are under-expressed or single enzymes are not functional (González-Rovira et al., 2009; Izquierdo et al., 2008; Robin and Skalli, 2007; Tocher and Ghioni, 1999). Thus, marine fish are dependent on dietary supply with EPA and DHA and thus have a high dietary demand for these n-3 HUFAs (NRC, 2011; Sargent et al., 2002).

Aquafeeds and current constrains

As a result, aquafeeds for marine species differ from those for freshwater fish with regard to the fatty acid composition and amounts of EPA and DHA. Obviously, the choice of oil source is important for adequate nutrition of farmed fish. The richest and readily available source of EPA and DHA is marine fish oil and the oil fraction in marine fish meal (Gunstone, 2011). The traditionally high amounts of fish meal and especially fish oil for both, marine and freshwater species have been decreased due to the reduced market availability, increasing prices and the demand for a sustainable aquaculture (Shepherd and Jackson, 2013). Consequently, the amount and variety of terrestrial oil sources has been elevated in aquafeeds, resulting in increased amounts of n-6 fatty acids and short chained fatty acids. Many fish species are able to grow unhampered on diets with increased amounts of vegetable oils, fresh water fish better than marine fish. A drawback is the resulting reduced product quality of farmed fish (FAO, 2014; Shepherd and Jackson, 2013; Sprague et al., 2016). If nutrition of farmed fish continues as is, we will soon farm fish that have increasingly similar n-6/n-3 PUFA ratios to terrestrial stocks and thus are about to lose their unique role in human nutrition.

Future goals

Future goals for fish nutrition and aquaculture should thus be an increase in n-3 PUFAs in farmed fish products to improve the n-6/n-3 ratio and produce healthy fish suitable for a high-quality human nutrition. The main task would be the balance between sustainable aquafeeds with low levels of fish meal and fish oil, but sufficient n-3 PUFA supply for the fish (Tocher, 2015). Besides attempts focusing on the use of novel feed ingredients and alternative EPA and DHA sources (Betancor et al., 2015; Miller et al., 2011; Shah et al., 2017), the stimulation of the *in vivo* fatty acid synthesis is of great interest. The majority of farmed fish worldwide belong to the freshwater lifestyle (e.g. cyprinids and tilapia, (FAO, 2014)) and thus have a natural capacity to synthesize EPA and DHA. An interaction with the molecular pathway of the *in vivo* fatty acid biosynthesis is an innovative possibility to regulate the fatty acid composition of the fish without relying on natural EPA and DHA sources.

In the case of freshwater fish, a stimulation of the endogenous fatty acid synthesis holds great potential. The choice of dietary oil source and fatty acid composition may already influence the efficiency of the fatty acid synthesis. Whereas dietary EPA and DHA hamper the synthesis, dietary ALA would stimulate it (Gregory et al., 2016). Moreover, fatty acids from the n-6 and omega-9 (n-9) series are able to interact with the conversion of ALA to EPA and DHA, since the same enzymes are simultaneously involved in the biosynthesis of fatty acids from the n-3, n-6, and n-9 series (Sargent et al., 2002). In order to maximize the potential of freshwater fish to synthesize EPA and DHA *in vivo*, dietary oil sources rich in ALA should be favored. Furthermore, an improvement of the rather slow natural fatty acid bioconversion of ALA to EPA and DHA should be emphasized.

In marine fish, nutritional regulation in order to stimulate the fatty acid biosynthesis has so far been partly successful (González-Rovira et al., 2009; Robin and Skalli, 2007; Xue et al., 2015). As mentioned above, the fatty acid synthesis in marine fish is disrupted, although the genetic capacity is expected to be present. Some marine fish are able to partly perform fatty acid synthesis. The challenge in marine species would be, to activate the dormant genes encoding enzymes involved in fatty acid synthesis that are still present within the fish and ensure an entire fatty acid synthesis.

Functional and bioactive substances

Secondary plant compounds with functional and bioactive properties might hold the key for an increase in n-3 HUFAs in fish *via* a stimulation of the *in vivo* fatty acid synthesis. Numerous bioactives have been investigated in past years based on their expected potential to increase EPA and DHA in different animals (reviewed in Rupasinghe

et al. (2016). The substances of interest for this thesis are the stilbene resveratrol and the phytoestrogen genistein.

Previous studies have indicated that resveratrol is able to increase the levels of n-3 HUFAs like EPA and DHA in different animal models and interact with the lipid metabolism pathway (Caro et al., 2017; Frémont et al., 1999; Momchilova et al., 2014). The underlying molecular mechanisms how resveratrol might interact with the endogenous fatty acid synthesis of fish are yet to be clarified. There is reasonable evidence that genistein affects the lipid metabolism in different fish species, including alterations of plasma triglyceride and cholesterol levels (D'Souza et al., 2005; Dias et al., 2005; Rupasinghe et al., 2016; Schiller Vestergren et al., 2011). Both substances hold great potential to stimulate the *in vivo* fatty acid biosynthesis in fish.

Contribution of this work to current knowledge

Based on the presented background, four research chapters investigating effects of resveratrol and genistein in the nutrition of freshwater and marine fish are included in this thesis. Rainbow trout (*Oncorhynchus mykiss*) was used as freshwater model species (**chapters I – III**) and gilthead sea bream (*Sparus aurata*) as model species with a marine lifestyle (**chapter IV**). The majority of the following chapters deal with results obtained in trials with rainbow trout. It was hypothesized that their precondition to perform the fatty acid synthesis would enable a better understanding of underlying molecular mechanisms. The sea bream trial focused on the potentially more promising substance resveratrol. Results concerning the use of genistein in sea bream nutrition are yet to be fully understood and further analyses will be conducted postdoctoral.

Chapter I focuses on the first-time use of resveratrol in diets for rainbow trout and addresses the following research questions:

- Does dietary resveratrol change the fatty acid composition of rainbow trout?
- Is the hepatic mRNA expression of genes influenced by dietary resveratrol?
- Does dietary resveratrol alter the hepatic enzyme levels of the key desaturase in fatty acid biosynthesis?
- Does the dietary fish oil level play a role in the effectiveness of resveratrol?

Chapter II examines the following research questions about the use of genistein and resveratrol in diets for rainbow trout:

- Does dietary genistein affect the fatty acid composition of rainbow trout?
- How do dietary genistein and resveratrol influence the growth, nutrient utilization, and performance of rainbow trout?
- Are dietary genistein and resveratrol able to modify the whole body nutrient composition of rainbow trout?
- Which role does the dietary fish oil level play in the response of the investigated parameters to the dietary bioactives?

Chapter III is based on the observations made within chapter I and chapter II and highlights the following topics:

- Does the digestibility of macronutrients in rainbow trout respond to dietary genistein and resveratrol?
- Can dietary genistein and resveratrol lead to histopathological alterations in the hindgut of rainbow trout?

Chapter IV addresses several research questions on the use of resveratrol in diets for gilthead sea bream:

- What are the effects of dietary resveratrol on the fatty acid composition of different tissues of sea bream?
- Are the hepatic mRNA levels of genes encoding proteins involved in fatty acid biosynthesis and metabolism affected by dietary resveratrol?
- Does dietary resveratrol influence the growth, performance, and nutrient composition of sea bream?
- Which role does the dietary fish oil level play in the response of the investigated parameters?
- Does the rearing temperature affect the outcome of the analyzed parameters?

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CHAPTER I

Fatty acid profile is modulated by dietary resveratrol in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

To produce fish of a high quality that are rich in omega-3 fatty acids (n-3 FA) and simultaneously generate more sustainable aquaculture, the combined use of phytochemicals and vegetable oils in fish feed seems to be a promising approach. Resveratrol (RV) potentially induces endogenous fatty acid synthesis, resulting in elevated n-3 FA levels in fish. RV putatively influences Δ6-desaturase, the key enzyme in FA metabolism, and serves as a ligand for PPARα, a transcription factor regulating β-oxidation. Rainbow trout (36.35 ± 0.03 g) were randomly allocated into six groups and fed diets with reduced fish oil levels (F4 = 4%, F2 = 2% and F0 = 0% of dry matter) supplemented with 0.3% (*w*/*w*) RV (F4 + RV, F2 + RV and F0 + RV). RV significantly affected FA composition in liver tissue and whole fish homogenates. 20:5n-3 (EPA) and 22:6n-3 (DHA) were significantly increased whereas precursor FA were diminished in fish fed the F2 + RV and F0 + RV diets when compared to F4 + RV and F0. RV significantly elevated Δ6-desaturase protein levels in the livers of F0 + RV fed animals. Hepatic mRNA expression of Δ6-desaturase, PPARα, and its target genes were affected by the dietary fish oil level and not by dietary RV. The results of this study indicated a potential benefit of supplementing RV in fish oil deprived diets elevating n-3 FA levels in rainbow trout.

Keywords: CPT1; fish oil replacement; hepatic fatty acid synthesis; long chain polyunsaturated fatty acids; mRNA expression; phytochemical; PPARα

1. Introduction

Seafood, fish, and fish products are important dietary sources of health beneficial omega-3 fatty acids (n-3 FA) and thus important in human nutrition (FAO, 2010; Harris et al., 2009; WHO, 2003; Williams and Burdge, 2006). In particular the n-3 long chain poly-unsaturated FA (LC-PUFA) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) may mediate health benefits and are of increasingly recognized importance (Baker et al., 2016; de Lorgeril and Salen, 2012; Molendi-Coste et al., 2011; Tapiero et al., 2002). For the production of healthy fish rich in these n-3 LC-PUFAs, the nutrition of the fish itself plays a crucial role (Tocher and Sargent, 1990). On the one hand, marine ingredients in fish diets have a positive effect on fish health, survival and growth rates (Caballero et al., 2002; Montero et al., 2003). These are often plant based and differ in nutritional value, digestibility and nutrient availability in comparison to marine derived feed ingredients. When dietary fish oil is replaced by plant oils, the resulting FA composition in fish often changes to a profile rather poor in n-3 FA, thus making them less valuable for human nutrition (Bell et al., 2001b, 2003; FAO, 2014; Regost et al., 2003; Shepherd and Jackson, 2013; Sprague et al., 2016). On the other hand, the shortage of marine resources for fish meal and fish oil production and the demand for sustainable aquaculture requires the use of alternative raw materials. Considerable effort has been made in the past decade to counteract this trend where various approaches have been considered including the use of n-3 FA rich finishing diets after feeding plant based diets (Regost et al., 2003; Turchini et al., 2011), the inclusion of microalgae rich in n-3 FA (Betiku et al., 2016; Haas et al., 2015; Sprague et al., 2015), the use of n-3 enriched oil from genetically modified oilseeds (Betancor et al., 2015), and the application of secondary plant compounds to alter endogenous n-3 FA synthesis (Schiller Vestergren et al., 2012; Trattner et al., 2008a), amongst others.

Fresh water fish have the ability to synthesize EPA and DHA (Sargent et al., 1999, 2002; Tocher, 2003) from the essential precursor n-3 PUFA alpha-linolenic acid (ALA, 18:3n-3). The hepatic FA conversion from ALA to EPA and further to DHA takes place in the endoplasmic reticulum and comprises several elongation and desaturation steps, followed by a final β-oxidation in the peroxisome (Burdge, 2004; Sargent et al., 2002) (Figure I- 1). The LC-PUFA synthesis is a complex process requiring different enzymes, transcription factors and various target genes. The rate-limiting key enzyme of this process is Δ6-desaturase (Δ6-D), which introduces an additional double bond in ALA and initiates the LC-PUFA synthesis (Vagner and Santigosa, 2011). Aside from the Δ6-D and Δ5-desaturase (Δ5-D), the nuclear hormone receptors peroxisome proliferator-activated receptors (PPARs) and its target genes including carnitine palmitoyl transferase 1 (CPT1),

sterol regulatory element binding protein 1 (SREBP-1), and acyl-CoA oxidase 1 (acox1) are centrally involved in LC-PUFA synthesis and regulation of this process (Figure I- 1).

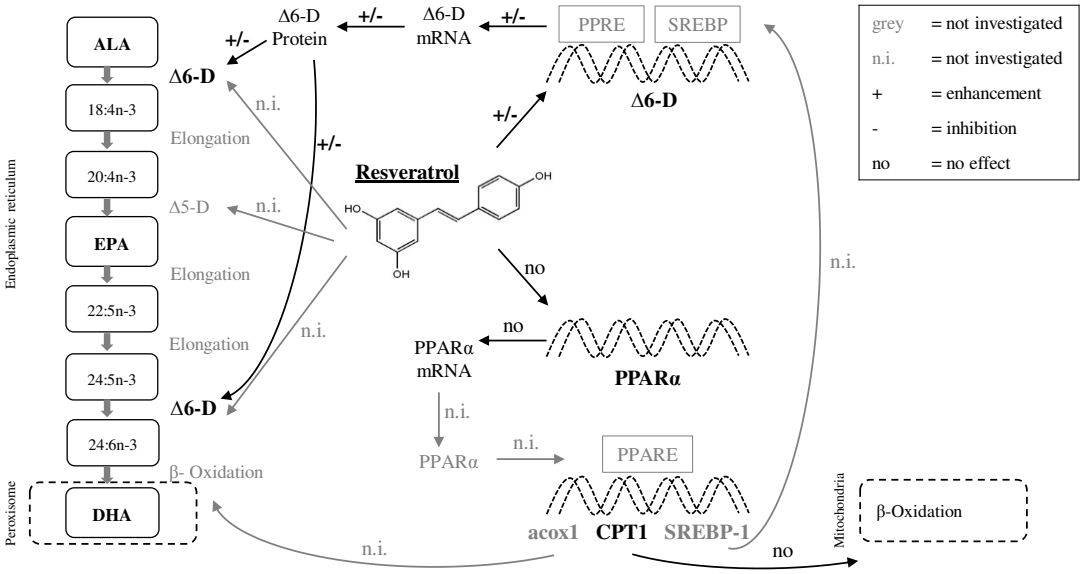

Figure I- 1. Molecular pathways of the hepatic endogenous fatty acid synthesis in the endoplasmic reticulum and peroxisomes and putative effects of resveratrol (RV) on the expression level and activity of involved enzymes and transcription factors. Alpha-linolenic acid (ALA) is converted to eicosapentaenoic acid (EPA) and further to 24:6n-3 *via* elongation and desaturation (Δ6-desaturase (Δ6-D) and Δ5-desaturase (Δ5-D)) steps (Sargent et al., 2002). The partial β-oxidation to form docosahexaenoic acid (DHA) takes place in peroxisomes. RV possibly affects Δ6-D and Δ5-D activity, or interacts with Δ6-D *via* transcriptional control of gene expression (Momchilova et al., 2014). Further, RV putatively enhances the peroxisome proliferator-activated receptor α (PPARα) gene expression (Inoue et al., 2003; Tsukamoto et al., 2010). PPARα might induce the expression of target genes, for example carnitine palmitoyl transferase 1 (CPT1), sterol regulatory element binding protein 1 (SREBP-1) and acyl-CoA oxidase 1 (acox1) (Schiller Vestergren et al., 2011; Trattner et al., 2008b), putatively increasing mitochondrial β-oxidation (CPT1), peroxisomal β-oxidation (acox1) and Δ6-D gene expression (SREBP-1). Graph made according to Sargent et al. (2002) and Burdge(2004) and modified according to results from previous studies (Inoue et al., 2003; Momchilova et al., 2014; Schiller Vestergren et al., 2011; Trattner et al., 2008b; Tsukamoto et al., 2010) and own data.

In rainbow trout (*Oncorhynchus mykiss*), the endogenous FA synthesis may last 2-4 weeks to convert dietary ALA into EPA and DHA and contributes only to a small extend to n-3 LC-PUFA tissue levels (Bell et al., 2001a). This indicates that LC-PUFAs are putatively essential for rainbow trout and should be supplied *via* dietary intake. Under experimental conditions, it is feasible to reduce marine feed ingredients (and thus dietary EPA and DHA levels) without compromising fish performance (Richard et al., 2006; Thiessen et al., 2004). However, a more recent study showed that a complete reduction of marine feed ingredients over a whole production cycle in rainbow trout was possible (Lazzarotto et al., 2015). However, the vegetable diet significantly negatively affected

growth and the n-3 FA content of fish (carcass n-3 PUFAs in polar lipids reduced from 45.1% to 32.1% fatty acid methyl esters (FAMEs) and in neutral lipids from 31.4% to 14.3% FAMEs) (Lazzarotto et al., 2015). Thus, in commercial scale production, trout diets still need to meet minimum requirements for essential fatty acids (EPA + DHA: 0.4–0.5% dry matter (DM) of diet and ALA: 0.7-1.0% DM of diet (NRC, 2011)) to ensure an end product with adequate amounts of n-3 LC-PUFA. According to several studies, the n-3 LC-PUFA content of farmed and wild salmonids varies largely depending on commercial feed and/or region (rainbow trout farmed versus wild: 931 and 268 mg/100 g, respectively (Blanchet et al., 2005); Atlantic salmon farmed vs. wild: >1400 and <900 mg/100 g, respectively (Sprague et al., 2016)). In farmed salmonids, amounts of EPA and DHA are decreasing parallel to increasing use of dietary vegetable oils leading to increased portion sizes to meet recommended dietary amounts of EPA + DHA of 200-500 mg/day (ISSFAL, 2004; Sprague et al., 2016). For the production of healthy fish with sufficient amounts of EPA and DHA and a reduction of marine dietary resources, secondary plant compounds that may have the ability to modify endogenous FA synthesis *in vivo* should be considered. Genistein and lipoic acid (Schiller Vestergren et al., 2011), or a mixture of episesamin/sesamin (Schiller Vestergren et al., 2011; Trattner et al., 2008b) were shown to increase the expression levels of genes encoding proteins involved in FA synthesis in salmon hepatocytes *in vitro* (for example Δ6-D, PPARα and CPT1). Further, Trattner et al. (2008a) enhanced EPA and DHA levels in rainbow trout *in vivo* with feeding diets containing a sesamin/episesamin mixture. EPA was elevated significantly from 5.2% to 5.6% FAMEs and DHA from 38.6% to 43.7% FAMEs when rainbow trout were fed sesamin/episesamin-supplemented diets (Trattner et al., 2008a).

In this study, the phytochemical resveratrol (RV) was investigated for its potential to induce LC-PUFA synthesis in rainbow trout. RV is a stilbene found in different plants, mainly grapevine, with potentially health-beneficial and, amongst others, anti-oxidant and anti-inflammatory properties (Brisdelli et al., 2009; Cho et al., 2017; Soleas et al., 1997). Over thirty years ago, RV was proven to modulate lipid metabolism (reduced lipogenesis and increased lipolysis) in rats (Arichi et al., 1982; Kimura et al., 1983). RV up-regulated PPARα in a neuron model (Cheng et al., 2009) and furthermore was described as PPAR activator in several *in vitro* and *in vivo* studies, as reviewed by Nakata et al. (2012). Two studies, both combining *in vitro* cell culture assays with *in vivo* experiments in mice, suggested that RV activated PPARα (Inoue et al., 2003; Tsukamoto et al., 2010). RV putatively increased elongase and desaturase activities (Δ6-D and Δ5-D) resulting in elevated DHA levels (Momchilova et al., 2014) (Figure I- 1). Recently, polyphenol-rich wine lees extracts were shown to modulate FA metabolism and FA profile in zebra fish embryos (Caro et al., 2017). To the best of our knowledge, this is the first study

investigating the potential effects of RV on endogenous FA synthesis in rainbow trout *in vivo*.

To investigate how the phytochemical RV may affect n-3 LC-PUFA synthesis, feeding experiments with rainbow trout were conducted. Experimental diets with RV supplementation and differing fish oil inclusion levels and thus, varying dietary LC-PUFA contents were used. The resulting FA composition of liver tissue and whole body homogenates, the hepatic mRNA steady state levels of Δ6-D, PPARα, CPT1a and CPT1c, and hepatic protein levels of Δ6-D were determined.

2. Materials and Methods

2.1. Experimental diets and housing conditions of rainbow trout

Six different experimental diets (isonitrogenous and isoenergetic) were formulated on dry matter (DM) basis as shown in Table I- 1. All diets contained 10% of fish meal and consisted of equal amounts of mainly alternative plant protein sources (pea protein isolate, wheat gluten and rapeseed concentrate amongst others; Table I- 1). The experimental diets varied in the oil fraction, with decreasing fish oil contents (F4: 4%, F2: 2% and F0: 0% DM) and increasing vegetable oil contents (F4: 6.6%, F2: 8.6% and F0: 10.6% DM). Diet F4 served as an overall reference diet and accordingly as the control. Diet F2 contained half the amount of fish oil of diet F4 (50% substitution with plant oils) and was set at the requirement level of EPA + DHA in trout (0.4–0.5% DM of the diet (NRC, 2011)). Diet F0 completely lacked fish oil (100% substitution with plant oils) and served as the reference for a diet below the minimum requirement of EPA + DHA in rainbow trout. Fatty acid profiles and absolute amounts of EPA + DHA in % DM of experimental diets are presented in Table I- 2. The three above-mentioned diets were referred to as basal diets (F4, F2, F0) and served as controls for similarly composited, but additionally with resveratrol (RV; *trans*-3,4′,5-trihydroxy stilbene, purity min. 98%, CHEMOS GmbH & Co. KG, Regenstauf, Germany) supplemented diets. Diets F4 + RV, F2 + RV and F0 + RV were supplemented with RV (0.3% DM, Table I- 1). The amino acid (AA) content of each diet was calculated based on the AA contents of single ingredients. All diets were formulated to meet the requirements for AA content in rainbow trout feed according to Rodehutscord et al. (1997) and NRC (2011).

A feeding trial with a total of 486 juvenile rainbow trout (Fischzucht Kortmann GbR, Hohenweststedt, Germany) was conducted at the facilities of the Gesellschaft für Marine Aquakultur (GMA) mbH in Büsum, Germany. All experiments were carried out according to the EU Directive 2010/63/EU for animal experiments and approved by the Ministry of Energy, Agriculture, the Environment, Nature and Digitalization (MELUND), Kiel, Germany

(approved on 15 October 2014; project number: V244-7224.121.9-34). Trout were allowed to adapt to housing conditions prior to the experiment in a recirculating aquaculture system (7.6 m^3, turnover rate 4 times h^{-1}, moving bed bio filter and additional bead filter (PolyGeyser, Model DF-6, Aquaculture Systems Technologies, L.L.C., New Orleans, LA, USA), UV-light disinfection). Light regime was set at 14:10 h light:dark cycle during adaption and experimental treatment periods. Water quality parameters were determined daily and maintained in a suitable range for rainbow trout (7.93 pH, 15.1 ± 0.4 °C, 9.4 ± 0.3 mg L^{-1} O_2, 0.2 ± 0.1 mg L^{-1} NH_4, 6.9 ± 3.4 mg L^{-1} NO_2 (Microquant test kit for NH_4 and NO_2, Merck, Darmstadt, Germany), 2.3 ± 0.7‰ PSU).

Table I- 1. Ingredients and nutrient composition (in % of dry matter (DM)) of the experimental diets. F4, F2 and F0 are basal diets containing 4%, 2% and 0% DM fish oil. +RV indicates supplementation of basal diets with 0.3% DM resveratrol.

Ingredients [% DM]	F4	F2	F0	F4 + RV	F2 + RV	F0 + RV
Fish meal (Clupea sp.) [a]	10	10	10	10	10	10
Casein [b]	5.9	5.9	5.9	5.9	5.9	5.9
Rapeseed concentrate [c]	13	13	13	13	13	13
Pea protein isolate [d]	13.58	13.58	13.58	13.58	13.58	13.58
Wheat gluten [e]	17.95	17.95	17.95	17.95	17.95	17.95
Wheat starch [e]	20	20	20	20	20	20
Fish oil [a]	**4**	**2**	**-**	**4**	**2**	**-**
Linseed oil [f]	1.50	0.94	0.38	1.50	0.94	0.38
Rapeseed oil [g]	3.19	6.11	9.04	3.19	6.11	9.04
Sunflower oil [g]	1.91	1.55	1.18	1.91	1.55	1.18
Vitamin mineral premix [h]	0.5	0.5	0.5	0.5	0.5	0.5
Lysine [i]	0.7	0.7	0.7	0.7	0.7	0.7
Dicalcium-phosphate [j]	1	1	1	1	1	1
Inert filler [k]	6.77	6.77	6.77	6.77	6.77	6.77
Resveratrol (RV) [l]	-	-	-	**0.3**	**0.3**	**0.3**
Nutrient composition [% DM]						
Dry matter (in % of diet)	93.2	93.1	93.2	93.3	92.8	91.7
Crude protein	50.1	50.3	50.2	50.0	50.3	50.8
Crude lipid	14.9	15.1	15.1	15.0	15.0	15.0
Crude ash	6.4	6.4	6.4	6.4	6.4	6.2
Total carbohydrates [m]	28.6	28.2	28.4	28.6	28.3	28.0
Gross energy [MJ kg^{-1} DM]	22.72	22.72	22.70	22.75	22.75	22.93

[a] Vereinigte Fischmehlwerke Cuxhaven GmbH & Co. KG, Cuxhaven, Germany; [b] Molkerei MEGGLE Wasserburg GmbH & Co. KG, Wasserburg, Germany; [c] BioExx Speciality Proteins LTD, Toronto, ON, Canada; [d] Emsland-Stärke GmbH, Emlichheim, Germany; [e] KRÖNER STÄRKE GmbH, Ibbenbüren, Germany; [f] Makana Produktion und Vertrieb GmbH, Offenbach a.d. Queich, Germany; [g] Different food stores, Büsum, Germany; [h] Emsland-Aller Aqua GmbH, Golßen, Germany; [i] Biolys, Evonik Industries AG, Essen, Germany; [j] Lehmann & Voss & Co (LuV), Hamburg, Germany; [k] Carboxy-methyl-cellulose (CMC), Mikro-Technik GmbH & Co. KG, Bürgstadt/Main, Germany; [l] CHEMOS GmbH & Co. KG, Regenstauf, Germany; [m] Total carbohydrates = 1000 − (crude protein + crude fat + crude ash).

Table I- 2. Fatty acid composition (in % of total fatty acid methyl esters (FAMEs) and in % DM of diet) of the experimental diets. F4, F2 and F0 are basal diets containing 4%, 2% and 0% DM fish oil. +RV indicates supplementation of basal diets with 0.3% DM resveratrol. The standard used for identification of individual FAMEs consisted of all 11 FAMEs shown here.

[% of FAMEs]	F4	F2	F0	F4 + RV	F2 + RV	F0 + RV
14:0	1.79	1.04	0.70	1.77	1.04	0.35
16:0	11.24	9.75	9.76	11.22	9.74	8.89
18:0	3.58	3.18	3.17	3.55	3.11	2.95
Σ SFA [a]	**16.60**	**13.97**	**13.64**	**16.54**	**13.90**	**12.20**
16:1	1.72	1.09	0.83	1.73	1.08	0.52
18:1n-9c	33.46	40.03	42.00	33.38	39.67	45.18
18:1n-7c	2.65	2.92	3.14	2.68	2.99	3.21
Σ MUFA [b]	**37.83**	**44.04**	**45.95**	**37.79**	**43.74**	**48.91**
18:2n-6c	28.83	28.26	27.90	28.87	28.61	27.79
18:3n-3	10.95	10.11	9.29	11.00	10.07	8.93
20:5n-3	2.28	1.44	1.20	2.35	1.40	0.68
22:5n-3	0.49	0.18	0.27	0.32	0.33	0.21
22:6n-3	3.02	2.00	1.80	3.12	1.96	1.27
Σ PUFA [c]	**45.57**	**41.99**	**40.42**	**45.67**	**42.37**	**38.89**
Σ EPA + DHA [d]	**5.30**	**3.43**	**2.35**	**5.47**	**3.36**	**1.96**
EPA/DHA	0.75	0.72	0.61	0.75	0.72	0.54
18:3n-3/18:2n-6	0.38	0.36	0.33	0.38	0.35	0.32
Σ EPA + DHA % DM [e]	**0.74**	**0.56**	**0.34**	**0.91**	**0.56**	**0.26**

[a] ∑ SFA is the sum of saturated fatty acids; [b] ∑ MUFA is the sum of monounsaturated fatty acids; [c] ∑ PUFA is the sum of n-3 and n-6 polyunsaturated fatty acids; [d] ∑ EPA + DHA is the sum of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3); [e] Determination of EPA + DHA % DM of diet was done using the internal standard 13:0 methyl ester and amount of lipid measured in the diet (Table I-1).

Prior to experimental treatment, rainbow trout (mean body weight 36.35 ± 0.03 g) were randomized in six different groups in triplicate, each group with 27 individuals, and maintained in 18 tanks (150 L) of the experimental system. During the experimental period of eight weeks, fish were fed manually twice per day (8:30 a.m. and 4:30 p.m.) until apparent satiation.

2.2. Sampling

Samples were collected before the onset of the experimental period (day 0) and at the end of the experiment (day 58). For initial sampling at day 0, five individuals were sacrificed (pooled sample) and stored at −20 °C for determination of whole body fatty acid

composition. In addition, five individuals were sacrificed for collection of liver samples. For mRNA quantification *via* qRT-PCR, one part of the liver tissue was preserved in RNALater (Sigma-Aldrich, Taufkirchen, Germany) and stored at −20 °C. For measurements of the protein levels *via* ELISA, a second part of the liver tissue was immediately shock-frozen and stored at −80 °C. For the determination of the liver lipid levels and the liver fatty acid composition, the remaining liver parts of five individuals were pooled into one sample and immediately stored at −80 °C. At the end of the feeding trial at day 58, corresponding samples for the determination of mRNA and protein levels were taken from five individuals per tank. Additionally, five individuals per tank were sacrificed (pooled samples) for analysis of whole body fatty acid composition.

2.3. Lipid extraction and measurement of fatty acids

Extraction of total lipids and measurement of fatty acid methyl esters (FAMEs) was performed using a Gas Chromatograph with Flame Ionization Detector (GC-FID).

In brief, total lipids were extracted from liver samples according to Folch et al. (1957). Methylation of fatty acids and extraction of methylated FAMEs was conducted with the help of the Folch reagent (chloroform:methanol 2:1). Samples were neutralized using potassium hydroxide (0.1 M) and FAMEs were isolated by the addition of the Folch reagent and subsequent centrifugation for 10 min at 2000× *g*. The organic phase was collected and a second extraction with potassium hydroxide and the Folch reagent was performed, followed by centrifugation (5 min at 2000× *g*) and drying of samples under a N_2 flux. Re-dissolved FAME samples were injected into a 7820A Agilent gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent HP-88 fused silica capillary column (60 m × 250 µm × 0.2 µm, Agilent Technologies) and helium (1.2 mL/min) as the carrier gas. The following temperature protocol was applied: initial temperature 125 °C, ramp 8 °C/min to 145 °C (26 min), ramp 2 °C/min to 220 °C (5 min). Chromatograms were recorded and analyzed using EZChrom Elite software (Agilent Technologies). FAME standards (11 FAMEs, see Table I- 2) were used to identify retention times of individual FAMEs. Fatty acid composition was calculated as a percentage of single FAME relative to total FAMEs. FAs as % DM of diet or mg/g tissue were calculated using 13:0 methyl ester as the internal standard.

2.4. mRNA extraction and qRT-PCR

Total mRNA was extracted from the trout liver samples using the Innuprep RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol. Tissue was homogenized in a TissueLyser II (Qiagen, Hilden, Germany) prior to RNA isolation. RNA concentration and purity were determined *via* NanoDrop measurements (NanoDrop2000c;

ThermoScientific, Waltham, MA, USA) at 260, 280 and 230 nm absorbance. qRT-PCR was performed with a SensiFast SYBR No-ROX One-Step Kit (Bioline, London, UK) on a Rotor-Gene 6000 real-time PCR cycler (Corbett/Qiagen). Primers used and appropriate annealing temperatures are listed in Table I-3. Transcript expression was absolutely quantified by calculating the input copy number using a standard curve. Subsequently, respective target mRNA expression levels of Δ6-D, PPARα, CPT1a and CPT1c were normalized to the mRNA expression level of the housekeeping gene elongation factor 1 α (EF1α). Data are shown as relative mRNA expression levels of respective target genes normalized to their internal control (EF1α) following absolute quantification (Figure I- 2).

Table I- 3. Primer sequences for hepatic mRNA measurements *via* qRT-PCR. Forward and reverse primers as well as their specific annealing temperatures used for qRT-PCR measurements of mRNA levels in total RNA samples extracted from rainbow trout liver.

Primer	Sequence 5' → 3'	Annealing Temperature (°C)
Δ6-D [a,*] forward	GCTGGAGARGATGCCACGGA	61
Δ6-D [a,*] reverse	TGCCAGCTCTCCAATCAGCA	61
EF1α [b,*] forward	ACAAGCCCCTYCGTCTGCC	61
EF1α [b,*] reverse	GCATCTCCACAGACTTSACCTCAG	61
PPARα [c,§] forward	CTGGAGCTGGATGACAGTGA	55
PPARα [c,§] reverse	GGCAAGTTTTTGCAGCAGAT	55
CPT1a [d,§] forward	TCGATTTTCAAGGGTCTTCG	55
CPT1a [d,§] reverse	CACAACGATCAGCAAACTGG	55
CPT1c [d,§] forward	CGCTTCAAGAATGGGGTGAT	59
CPT1c [d,§] reverse	CAACCACCTGCTGTTTCTCA	59

[a] Δ6-D: Δ6-desaturase; [b] EF1α: Elongation factor 1 α; [c] PPARα: Peroxisome proliferator-activated receptor α; [d] CPT1: Carnitine palmitoyl transferase 1; * Geay et al. (2012); [§] Kolditz et al. (2008).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Determination of Δ6-D protein levels was performed in liver tissue samples from rainbow trout using a Fish Fatty Acid Desaturase 2 ELISA Kit (MBS066226, MyBiosource Inc., San Diego, CA, USA; purchased from Biozol, Eching, Germany) according to the manufacturer's instructions. In brief, tissue samples were weighted on dry ice and lysed in phosphate buffered saline using a TissueLyser II (Qiagen, Hilden, Germany). Following centrifugation, supernatants were applied to the MicroElisa multiwall plate provided by the Δ6-D Kit. Samples were incubated with a HRP (horseradish peroxidase)-conjugate reagent followed by several washing steps. Protein concentration was quantified following HRP-mediated color reaction (consecutive application of Chromogen A, B and Stop solutions) by absorbance measurements at 450 nm using a Labsystems iEMS MF

multiplate reader (MTX Lab Systems, Bradenton, FL, USA purchased from Thermo Fisher Scientific, Darmstadt, Germany). Δ6-D protein concentration was calculated using a standard curve. Δ6-D concentrations were normalized to total protein concentrations, which were evaluated *via* the Pierce bicinchoninic acid (BCA) kit (Thermo Fisher Scientific) according to the manufacturer's protocol, respectively.

2.6. Statistical analysis

All statistical analyses were performed using R (version 3.1.3) with an RStudio interface. The packages *gdata*, *multcomp*, *gplots*, *nparcomp*, *nlme*, and *piecewiseSEM* were used for graphical and statistical analysis.

For FA composition, liver lipid levels, initial body weight (IBW), final body weight (FBW), daily feed intake (DFI), Hepatosomatic index (HSI), and Δ6-D protein levels data evaluation started with the definition of an appropriate linear model. The data were proven to be normally distributed and homoscedastic based on a graphical residual analysis. The statistical model included the level of fish oil content (F4, F2, F0) and supplement (None, RV), as well as their interaction term. Based on this model, an analysis of variances (ANOVA) was conducted, followed by multiple contrast tests (Bretz et al., 2011; Schaarschmidt and Vaas, 2009) to compare several levels of influencing factors, respectively.

For mRNA expression levels, the data evaluation was initiated with the definition of an appropriate mixed model (Laird and Ware, 1982; Verbeke and Molenberghs, 2000) with fish oil content (F4, F2, F0), supplement type (None, RV) and their interaction term as fixed factors, and fish tank as random factor. A residual analysis revealed the data to be non-normally distributed. Multiple contrast tests for relative effects (Konietschke et al., 2012; Schaarschmidt and Vaas, 2009) were conducted to compare several levels of influencing factors, respectively.

3. Results

3.1. Resveratrol impairs body weight gain in rainbow trout

At the end of the feeding trial, the mean FBW of trout fed the un-supplemented basal diets (F4, F2, F0) was significantly higher compared to animals fed diets supplemented with RV (F4 + RV, F2 + RV and F0 + RV; Table I-4, $p < 0.05$ indicated by letters). The DFI was significantly ($p < 0.05$) reduced in trout fed the RV-supplemented diets compared to un-supplemented control diets. The HSI varied between 1.28 ± 0.30% (F2 + RV) and 1.52 ± 0.40% (F4) and did not significantly differ among groups.

Table I- 4. Growth, feed intake (DFI) and Hepatosomatic index (HSI) of rainbow trout fed the experimental diets. F4, F2 and F0 represent results which were obtained from fish fed the basal diets containing 4%, 2% and 0% DM fish oil. Basal diets were provided either in absence or presence of 0.3% DM resveratrol (+RV) for an 8-week feeding period.

	F4	F2	F0	F4 + RV	F2 + RV	F0 + RV
IBW [1]	35.54 ± 0.20	35.34 ± 0.15	35.69 ± 0.42	35.66 ± 0.15	35.81 ± 0.18	35.63 ± 0.50
FBW [2]	46.15 ± 1.26 [m]	46.37 ± 0.18 [x]	47.27 ± 2.19 [a]	41.96 ± 0.90 [n]	41.60 ± 1.15 [y]	40.57 ± 1.62 [b]
DFI [3]	1.94 ± 0.07 [m]	1.95 ± 0.06 [x]	1.90 ± 0.11 [a]	1.49 ± 0.04 [n]	1.49 ± 0.07 [y]	1.36 ± 0.08 [b]
HSI [4]	1.52 ± 0.40	1.49 ± 0.23	1.40 ± 0.16	1.32 ± 0.24	1.28 ± 0.30	1.51 ± 0.34

Values (mean ± SD, IBW, FBW and DFI: $n = 3$; HSI: $n = 5$) with different superscript letters within one row significantly differ with p-values < 0.05 based on ANOVA as described in Materials and methods. Superscript letters indicate the output of tests based on comparisons of supplement level within one fish oil inclusion group (a, b: F0 diets; x, y: F2 diets; m, n: F4 diets). [1] IBW = Initial body weight [g]; [2] FBW = Final body weight [g]; [3] DFI = Daily feed intake [% d^{-1}]; [4] HSI = Hepatosomatic index [%] = (liver weight/final body weight) × 100.

3.2. Experimental diets affect the fatty acid composition in whole body homogenates of rainbow trout

The FA composition of the diets (Table I- 2) was equally altered in the basal diets as in the corresponding RV supplemented diets with decreasing fish oil content. In general, monounsaturated FA (MUFA) content increased with decreasing fish oil content, while saturated FA (SFA) and PUFA contents decreased. Predominantly, the absolute amounts of EPA + DHA % DM of diet decreased with decreasing fish oil content from approximately 0.8% DM (F4 diets) to approximately 0.3% DM (F0 diets). At the same time, the ratio of ALA (18:3n-3) to LA (18:2n-6) remained unaffected (0.32–0.38) in all diets (Table I- 2).

The FA composition of the whole body homogenates of rainbow trout was not modified following a reduction of fish oil content while increasing the vegetable oil content in the basal diets (F4, F2 and F0; Table I- 5). Nevertheless, significant increases of EPA and DHA concentrations and simultaneous significant decreases of 18:2n-6 (LA) and 18:3n-3 (ALA) levels were only detected in whole fish homogenates when trout were fed the RV-supplemented diets (+RV; Table I- 5, $p < 0.05$ indicated by capital letters A, B). In RV-treated animals, tissue EPA + DHA levels significantly ($p < 0.05$) increased from 21.29 ± 7.29% (F4 + RV) to 32.63 ± 0.89% (F2 + RV) and 38.35 ± 3.38% (F0 + RV). The total amount of PUFA was also significantly ($p < 0.05$) increased from 38.41 ± 3.91% (F4 + RV) to 47.60 ± 1.55% (F0 + RV). The n-3/n-6 ratio was significantly ($p < 0.05$) increased from 2.34 ± 1.45 in fish fed the diet F4 + RV to 8.24 ± 2.61 in fish fed the F0 + RV diet.

Table I- 5. Fatty acid composition (in % of total fatty acid methyl esters (FAMEs)) of whole body homogenates of rainbow trout before (Initial) and at the end of the feeding trial. F4, F2, and F0 represent results which were obtained from fish fed the basal diets containing 4%, 2% and 0% DM fish oil. Supplementary designation with +RV indicates that fish were fed the diets supplemented with 0.3% DM resveratrol (+RV). The standard used for identification of individual FAMEs consisted of all 11 FAMEs shown here.

[% of FAMEs]	Initial	F4	F2	F0	F4 + RV	F2 + RV	F0 + RV
14:0	4.97	3.67 ± 0.29	3.29 ± 0.49	3.47 ± 0.26 ^{a}	3.19 ± 0.32 ^{A}	2.47 ± 0.19 ^{A,B}	1.73 ± 0.69 ^{b,B}
16:0	17.73	19.44 ± 0.84	20.15 ± 1.50	19.26 ± 0.52	18.54 ± 1.62	19.57 ± 0.47	20.16 ± 0.63
18:0	4.31	5.78 ± 0.48	6.26 ± 0.73	5.95 ± 0.47 ^{b}	5.73 ± 0.96 ^{B}	6.59 ± 0.34 ^{A,B}	7.96 ± 0.73 ^{a,A}
Σ SFA ^{a}	**27.01**	**28.89 ± 1.05**	**29.70 ± 2.09**	**28.68 ± 0.73**	**27.46 ± 2.71**	**28.63 ± 0.65**	**29.85 ± 0.71**
16:1	5.05	4.18 ± 0.52	3.70 ± 0.32 ^{x}	3.75 ± 0.29 ^{a}	3.50 ± 0.41 ^{A}	2.71 ± 0.13 ^{y,A,B}	2.15 ± 0.69 ^{b,B}
18:1n-9c	27.25	25.03 ± 0.91	23.94 ± 3.65	25.88 ± 1.77 ^{a}	27.46 ± 6.18	20.48 ± 0.53	17.91 ± 1.30 ^{b}
18:1n-7c	3.36	3.12 ± 0.02	3.10 ± 0.03 $^{+}$	3.17 ± 0.11 ^{a}	3.17 ± 0.21 ^{A}	2.8 ± 0.08 $^{A,+}$	2.48 ± 0.25 ^{b,B}
Σ MUFA ^{b}	**35.66**	**32.33 ± 1.34**	**30.74 ± 3.72**	**32.80 ± 2.11 ^{a}**	**34.13 ± 6.61 ^{A}**	**26.00 ± 0.61 ^{B}**	**22.55 ± 2.24 ^{b,B}**
18:2n-6c	14.11	11.81 ± 1.63	10.31 ± 2.67	11.41 ± 1.13 ^{a}	12.50 ± 3.44 ^{A}	8.72 ± 0.12 ^{A,B}	5.46 ± 1.63 ^{b,B}
18:3n-3	3.44	2.28 ± 0.35	1.92 ± 0.47	2.02 ± 0.21 ^{a}	2.66 ± 0.73 ^{A}	1.46 ± 0.10 ^{B}	0.97 ± 0.26 ^{b,B}
20:5n-3	4.29	3.91 ± 0.10	4.03 ± 0.63	3.74 ± 0.28 ^{b}	3.65 ± 1.13 ^{B}	4.68 ± 0.20 ^{A,B}	5.54 ± 0.41 ^{a,A}
22:5n-3	1.97	2.24 ± 0.05	2.28 ± 0.40	2.11 ± 0.12 $^{+}$	1.95 ± 0.71 ^{B}	2.57 ± 0.04 ^{A,B}	2.83 ± 0.21 $^{A,+}$
22:6n-3	13.53	18.55 ± 2.09	21.02 ± 3.88 $^{+}$	19.24 ± 2.33 ^{b}	17.65 ± 6.16 ^{B}	27.95 ± 0.84 $^{A,+}$	32.81 ± 3.11 ^{a,A}
Σ PUFA ^{c}	**37.34**	**38.78 ± 0.65**	**39.56 ± 1.74 ^{y}**	**38.52 ± 1.39 ^{b}**	**38.41 ± 3.91 ^{B}**	**45.38 ± 0.93 ^{x,A}**	**47.60 ± 1.55 ^{a,A}**
Σ EPA + DHA ^{d}	17.82	22.46 ± 2.19	25.05 ± 4.48	22.98 ± 2.60 ^{b}	21.29 ± 7.29 ^{B}	32.63 ± 0.89 ^{A}	38.35 ± 3.38 ^{a,A}
EPA/DHA	0.32	0.21 ± 0.02	0.19 ± 0.01 $^{+}$	0.20 ± 0.01 $^{++}$	0.21 ± 0.01 ^{A}	0.17 ± 0.01 $^{B,+}$	0.17 ± 0.01 $^{B,++}$
Σ n-3/Σ n-6	1.65	2.33 ± 0.45	3.03 ± 1.14	2.42 ± 0.57 ^{b}	2.34 ± 1.45 ^{B}	4.20 ± 0.07 ^{B}	8.24 ± 2.61 ^{a,A}

^{a} ∑ SFA is the sum of saturated fatty acids; ^{b} ∑ MUFA is the sum of monounsaturated fatty acids; ^{c} ∑ PUFA is the sum of n-3 and n-6 polyunsaturated fatty acids; ^{d} ∑ EPA + DHA is the sum of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3). Values (mean ± SD, n = 3, Initial: n = 1) with different superscript letters within one row significantly differ with p-values < 0.05 based on ANOVA as described in Materials and methods. Tests are based on comparison of supplement type within one fish oil inclusion level (a, b: F0 diets; x, y: F2 diets) or comparisons of fish oil inclusion level within +RV supplemented groups (A, B). $^{+/++}$ within one row indicates a statistical tendency between similarly marked groups with a p-value < 0.1.

When the FA profiles of rainbow trout that were provided with equal dietary fish oil levels, but either +RV or no supplementation with RV were compared, significant differences were most apparent in fish fed the fish oil free diets (F0 and F0 + RV). Concentrations of most FA changed in response to dietary supplementation with RV when compared to the controls (Table I- 5, $p < 0.05$ indicated by letters a, b and x, y). Most noticeably, DHA levels increased from 19.24 ± 2.33% in the controls (basal diet F0) to 32.81 ± 3.11% in the RV-treated animals (F0 + RV). Dietary supplementation with RV led to a significant ($p < 0.05$) increase in the contents of EPA + DHA, as well as total PUFA in the whole body homogenates of fish fed F2 + RV and F0 + RV diets. The n-3/n-6 ratio significantly ($p < 0.05$) increased from 2.42 ± 0.57 in fish fed the F0 diet to 8.24 ± 2.61 in fish fed the F0 + RV diet. No effect on FA profile was observed in groups which received the reference diets (F4 and F4 + RV).

3.3. Experimental diets affect lipid levels and fatty acid composition in livers of rainbow trout

The liver lipid levels (total fat mg/g) of rainbow trout fed the six experimental diets were altered by the dietary fish oil inclusion level and the dietary supplementation with RV (Table I- 6). The reduction of dietary fish oil from 4% DM to 0% DM led to a significant increase in the liver lipid levels (Table I- 6, $p < 0.05$ indicated by letters M, N). Furthermore, in the RV-treated animals, the liver lipid levels were significantly increased compared to the control animals (F4 + RV in comparison to F4 and F2 + RV in comparison to F2 (Table I- 6, $p < 0.05$ indicated by letters m, n and x, y, respectively).

The dietary treatment only slightly affected the relative FA profile (% FAMEs) in the livers of rainbow trout. The amount of SFA was decreased in fish fed the F0 + RV diet compared to the basal F0 diet (Table I- 6, $p < 0.05$ indicated by letters a, b), whereas the MUFA and the PUFA contents did not change based on dietary treatment. When the liver lipid levels are taken into account, the absolute amounts of EPA + DHA were significantly increased in fish that were fed the F0 diet compared to F4 (Table I- 6, $p < 0.05$ indicated by letters M, N). In the RV-treated fish, the absolute amounts of EPA, DHA and EPA + DHA were significantly ($p < 0.05$) elevated when fish were fed the F2 + RV and the F0 + RV diets compared to the un-supplemented basal diets. The absolute amounts of EPA + DHA were elevated from 8.69 ± 1.78 mg/g (F0) to 12.38 ± 2.93 mg/g (F0 + RV, Table I- 6, $p < 0.05$ indicated by letters a, b) in the liver tissues. No effect on FA profile was observed in groups which received the reference diets (F4 and F4 + RV).

Table I- 6. Total fat (mg/g liver) and fatty acid composition (in % of total fatty acid methyl esters (FAMEs) and in mg/g liver) of liver tissue of rainbow trout before (Initial) and at the end of the feeding trial. F4, F2, and F0 represent results which were obtained from fish fed the basal diets containing 4%, 2% and 0% DM fish oil. Supplementary designation with +RV indicates that fish were fed the diets supplemented with 0.3% DM resveratrol (+RV). The standard used for identification of individual FAMEs consisted of all 11 FAMEs shown here.

	Initial	F4	F2	F0	F4 + RV	F2 + RV	F0 + RV
Total fat [mg/g]	13.60	16.70 ± 1.56 ^{N,n}	21.47 ± 3.10 ^{x}	26.80 ± 2.55 ^{M}	24.80 ± 1.06 ^{m}	30.08 ± 3.11 ^{y}	26.70 ± 0.99
FA [% of FAMEs]							
14:0	1.63	1.45 ± 0.19	1.24 ± 0.13	1.33 ± 0.15	1.32 ± 0.08	1.4 ± 0.15	1.33 ± 0.11
16:0	20.75	22.80 ± 1.01	24.07 ± 1.35	24.30 ± 1.01 ^{a}	23.01 ± 0.6	22.89 ± 2.61	21.28 ± 0.71 ^{b}
18:0	6.90	8.14 ± 0.59	8.66 ± 0.58	8.15 ± 0.38	7.74 ± 0.38	7.52 ± 1.48	7.12 ± 0.18
Σ SFA ^{a}	**29.28**	**32.39 ± 1.22**	**33.96 ± 1.79**	**33.78 ± 1.11** ^{a}	**32.06 ± 0.89**	**31.81 ± 3.95**	**29.74 ± 0.79** ^{b}
16:1	2.20	1.64 ± 0.21	1.46 ± 0.25	1.92 ± 0.33	1.56 ± 0.25	1.91 ± 0.53	1.70 ± 0.32
18:1n9c	14.93	18.60 ± 4.28	15.14 ± 1.39	14.68 ± 1.00	14.58 ± 0.78	15.61 ± 1.24	16.40 ± 1.64
18:1n7c	2.37	2.55 ± 0.29	2.36 ± 0.23	2.66 ± 0.46	2.46 ± 0.33	2.72 ± 0.37	2.73 ± 0.29
Σ MUFA ^{b}	**19.50**	**22.79 ± 4.73**	**18.96 ± 1.86**	**19.25 ± 1.62**	**18.60 ± 1.23**	**20.25 ± 2.13**	**20.83 ± 2.19**
18:2n6c	8.17	6.09 ± 0.99	6.05 ± 0.99	5.74 ± 0.27	6.27 ± 0.62	5.73 ± 0.52	6.33 ± 0.35
18:3n3	1.45	0.69 ± 0.24	0.56 ± 0.16	0.47 ± 0.11	0.58 ± 0.08	0.57 ± 0.13	0.57 ± 0.20
20:5n3	4.59	3.90 ± 0.25	3.55 ± 0.37	3.65 ± 0.76	4.43 ± 0.41	4.37 ± 0.81	4.61 ± 0.64
22:5n3	1.87	1.87 ± 0.21 $^{+}$	1.88 ± 0.18	1.89 ± 0.22 ^{b}	2.30 ± 0.23 $^{+}$	2.22 ± 0.22	2.47 ± 0.19 ^{a}
22:6n3	35.13	32.26 ± 4.40	35.03 ± 1.48	35.21 ± 1.18	35.77 ± 0.93	35.06 ± 1.71	35.45 ± 1.89
Σ PUFA ^{c}	**51.21**	**44.81 ± 3.51**	**47.08 ± 0.21**	**46.97 ± 1.96**	**49.34 ± 0.72**	**47.95 ± 2.49**	**49.43 ± 1.79**
Σ EPA + DHA ^{d}	39.72	36.16 ± 4.50	38.58 ± 1.43	38.86 ± 1.68	40.20 ± 0.63	39.44 ± 2.46	40.06 ± 1.77
EPA/DHA	0.13	0.12 ± 0.02	0.10 ± 0.01	0.10 ± 0.02	0.12 ± 0.01	0.12 ± 0.02	0.13 ± 0.02
Σ n-3/Σ n-6	5.27	6.54 ± 1.67	6.92 ± 1.31	7.19 ± 0.45	6.92 ± 0.77	7.37 ± 1.03	6.83 ± 0.60
EPA mg/g	0.58	0.72 ± 0.24 $^{+}$	0.70 ± 0.03 ^{y}	0.83 ± 0.28 $^{b,+}$	1.02 ± 0.08	1.04 ± 0.39 ^{x}	1.29 ± 0.23 ^{a}
DHA mg/g	4.44	4.79 ± 1.19 ^{N}	7.00 ± 1.18 ^{y}	7.86 ± 1.52 ^{b,M}	8.25 ± 0.42	10.20 ± 0.68 ^{x}	11.08 ± 2.75 ^{a}
Σ EPA + DHA mg/g ^{e}	5.02	5.51 ± 1.23 ^{N}	7.71 ± 1.20 ^{y}	8.69 ± 1.78 ^{b,M}	9.27 ± 0.37	11.24 ± 0.78 ^{x}	12.38 ± 2.93 ^{a}

^{a} ∑ SFA is the sum of saturated fatty acids; ^{b} ∑ MUFA is the sum of monounsaturated fatty acids; ^{c} ∑ PUFA is the sum of n-3 and n-6 polyunsaturated fatty acids; ^{d} ∑ EPA + DHA is the sum of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3); ^{e} Determination of EPA + DHA mg/g liver tissue was done using the internal standard 13:0 methyl ester and amount of total fat in the liver. Values (mean ± SD, n = 3, Initial: n = 1) with different superscript letters within one row significantly differ with p-values < 0.05 based on ANOVA as described in Materials and methods. Tests are based on comparison of supplement type within one fish oil inclusion level (a, b: F0 diets; x, y: F2 diets; m, n F4 diets) or comparisons of fish oil inclusion level (M, N). $^{+}$ within one row indicates a statistical tendency between similarly marked groups with a p-value < 0.1.

3.4. Dietary fish oil content affects hepatic mRNA expression levels

The effect of dietary treatments (fish oil level and supplementation of plant bioactive compound) on mRNA expression levels of Δ6-D, PPARα, CPT1a and CPT1c were investigated in the rainbow trout livers (Figure I- 2). The reduction of fish oil content in the diet significantly increased the hepatic mRNA expression levels of Δ6-D in fish following the eight week feeding period (F4 compared to F2, Figure I- 2a, $p < 0.05$ indicated by *). Furthermore, hepatic PPARα mRNA expression levels were modulated by the different dietary fish oil levels (Figure I- 2b). Fish fed the F0 and F0 + RV diets displayed significantly ($p < 0.05$) lower PPARα mRNA expression levels when compared to fish fed the F2 and F2 + RV diets. This effect was independent of RV supplementation. Similar results were observed for the PPARα target gene expression CPT1c (Figure I- 2d). Hepatic mRNA expression of CPT1c was down-regulated when fish were fed the F0 diet in comparison to F2. When fish were fed diets supplemented with RV, the mRNA expression levels of CPT1c were significantly down-regulated in the group fed the F0 + RV diet when compared to F2 + RV and F4 + RV (Figure I- 2d, $p < 0.05$ indicated by *). Expression of CPT1a was neither influenced by dietary fish oil level nor dietary RV.

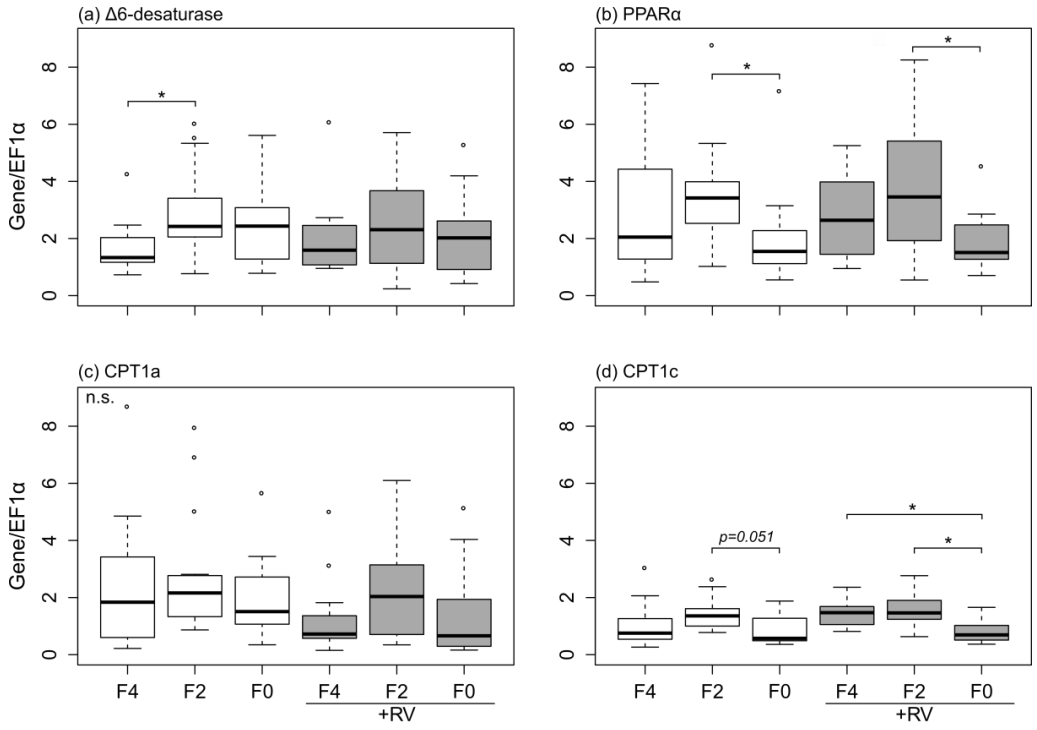

Figure I- 2. Hepatic mRNA expression levels in rainbow trout liver following dietary treatment with varying levels of fish oil and resveratrol supplementation for eight weeks. **(a)** Δ6-desaturase; **(b)** PPARα; **(c)** CPT1a and **(d)** CPT1c were measured in the liver of fish using qRT-PCR and were normalized to the housekeeping gene EF1α. Feeding groups F4, F2 and F0 were fed basal diets containing 4%, 2% and 0% DM fish oil, groups with +RV were fed respective diets supplemented with resveratrol. Boxes represent values (n = 15) between the 25 and 75 percentiles; whiskers indicate 1.5 SD; the solid line indicates the median; circles represent values above and below SD. Significant differences ($p < 0.05$) were analyzed using multiple contrast tests for relative effects. Tests were based on comparisons of fish oil inclusion level within one supplement group (indicated by *) or supplement type within one fish oil inclusion level (no significant differences).

3.5. Resveratrol affects hepatic ∆6-desaturase protein levels

Protein levels of ∆6-D were determined in the livers of rainbow trout fed different dietary fish oil levels and in the presence or absence of the dietary plant bioactive compound RV (Figure I- 3). Feeding the RV supplemented diets significantly affected hepatic ∆6-D protein levels in trout. ∆6-D protein levels were significantly increased in the livers of fish fed a diet completely lacking in fish oil (F0 + RV) when compared to fish fed the F4 + RV and F2 + RV diets (Figure I- 3, $p < 0.05$ indicated by *). The ∆6-D protein levels of fish fed the F0 + RV diet (0.823 ± 0.082 ng/mg protein) were equally high when compared to fish fed the control diet F4 (0.749 ± 0.145 ng/mg protein). Additionally, ∆6-D protein levels were significantly increased in the livers of fish fed the F0 + RV diet when compared to their F0 fed counterparts (Figure I- 3, $p < 0.05$ indicated by a, b). When dietary fish oil is 4%, RV even decreases the ∆6-D protein levels (F4 and F4 + RV, Figure I- 3, $p < 0.05$ indicated by x, y).

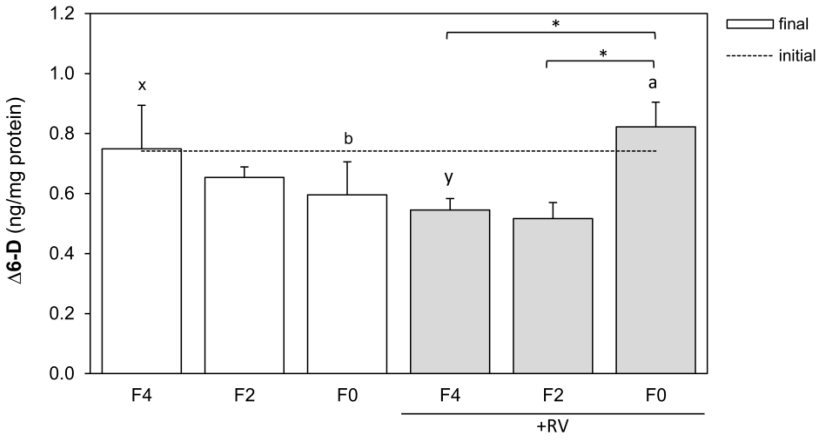

Figure I- 3. ∆6-desaturase (∆6-D) protein levels in the livers of rainbow trout following dietary treatment with varying levels of fish oil and resveratrol supplementation for eight weeks. The ∆6-D levels were measured using ELISA and were normalized to the total protein level (ng/mg protein). Feeding groups F4, F2 and F0 were fed basal diets containing 4%, 2% and 0% DM fish oil and groups +RV were fed respective diets supplemented with resveratrol. The dashed line indicates the initial expression value ($n = 1$), bars indicate the final expression values (mean + SD, $n = 3$). Statistically significant differences ($p < 0.05$) were analyzed using multiple contrast tests based on comparisons of fish oil inclusion level within one supplement group (*) or supplement type within one fish oil inclusion group (a, b: F0 diets; x, y: F4 diets).

4. Discussion

The discrepancy between (1) the need for constant high quality products with (2) the concurrent increasing demand for fish meal and fish oil for aquaculture production of fish for human nutrition accompanied by (3) the shortage of marine resources for fish oil production is a well-known yet unsolved problem. This study focused on the impact of

varying fish oil concentrations in fish diets (4%, 2% and 0% DM) in the absence or presence of the plant bioactive resveratrol (RV, 0.3% of DM) on n-3 long-chain PUFA synthesis in rainbow trout. The current study revealed positive effects of dietary RV on the PUFA content in the livers and the whole body homogenate of rainbow trout, including underlying molecular mechanisms.

The substitution of fish oil by vegetable oils and the provision of alternative, plant-derived protein sources in the fish diet is a common practice in salmonid and trout aquaculture. This “policy of replacement” has often caused alterations in the fatty acid composition in fish tissues (Bell et al., 2003; Drew et al., 2007; FAO, 2014; Fonseca-Madrigal et al., 2005; Shepherd and Jackson, 2013; Tocher, 2003) as dietary FA composition is strongly correlated to the fish tissue FA profile (Bell et al., 2001b; Caballero et al., 2002). Observations made during this study only partly agree with previous studies. SFA, MUFA and PUFA contents, as well as single FA concentrations varied within basal diets, but stayed consistent in the whole body homogenates of fish independent of diet (Table I- 5). Emery et al. (2013) showed that FA tissue composition did not necessarily reflect dietary FA composition, which may be related to an active *in vivo* FA metabolism. Furthermore, Sissener et al. (2016) suggested that it may be too simple to presume that the body FA profile reflects the dietary FA composition since different FA are diversely incorporated due to their suitability for functional capacities and energy supply. Moreover, the diet formulation used in this study was intentionally reduced in dietary EPA and DHA content (EPA + DHA levels in diets: 0.26–0.91% of DM of diet). In the above-mentioned studies, diets were always formulated to meet the requirements of EPA and DHA in trout (NRC, 2011). Comparison of the final and initial PUFA contents in fish tissue disclosed a reduction in LA, ALA and EPA concentrations (diets F4, F2 and F0). Simultaneously, an increase in DPA (22:5n-3) and DHA concentration was prevalent. This led to the assumption that the generally predominant vegetable based formulation of all diets evenly affected the n-3 FA synthesis regardless of the dietary fish oil level. Furthermore, trout fed a more vegetable based diet (F2) had significantly increased mRNA expression levels of Δ6-D compared to F4 fed animals. This was in accordance with previous studies on rainbow trout and salmonids (Caballero et al., 2002; Schiller Vestergren et al., 2012; Vagner and Santigosa, 2011). Nutritional modulation of activity and expression of Δ6-D has also been widely observed in teleost fish (Vagner and Santigosa, 2011) and in rainbow trout the maintenance of specific DHA levels was realized by the adaptation of desaturase activities (Caballero et al., 2002), especially when the diet lacked these LC-PUFA.

The dietary administration of resveratrol (+RV) resulted in a reduction of the final body weight (FBW), which was predominantly caused by a lower feed intake (DFI) in these fish

(Table I- 4). Since fish were fed to apparent satiation, the reduced DFI was an indication for lower appetite and earlier satiety. This effect was only visible in RV-treated groups and not the groups with reduced dietary fish oil (F2 and F0). Therefore, this indicates the lower palatability of RV-supplemented diets and no effect of dietary n-3 LC-PUFA levels in general. The lower palatability of fish diets may be caused by feed additives and secondary plant compounds as reviewed by Ajiboye et al. (2012). Growth depression and reduced feed intake as a result of dietary administration of secondary plant compounds has been described for Chinook salmon and rainbow trout (Bureau et al., 1998).

Nevertheless, the RV-supplementation of the experimental diets F2 and F0 (2% and 0% DM fish oil) significantly elevated EPA, DPA and DHA contents in trout tissue when compared to feeding with the respective basal diets. Simultaneously, ALA and LA levels were decreased in fish fed the +RV supplemented diets. An increase in EPA in the triglyceride fraction of zebra fish embryos in response to RV-rich wine lees had been reported by Caro et al. 2017 and partly supports the findings in this study. In a study with rainbow trout of similar size (IBW of 34 g) that were fed with diets lacking in fish oil, the dietary administration of the phytochemicals episesamin and sesamin increased relative tissue EPA and DHA contents (% FAMEs) to levels that are comparable to this study (Trattner et al., 2008b). In both studies, the EPA amount was increased to approximately 5.5% FAMEs, and the DHA amount was increased to approximately 43.8% FAMEs in the study by Trattner et al. (2008b) and to 32.8% FAMEs in this study. These results indicate a similar potential of RV in rainbow trout fed diets reduced in fish oil when set into the context described by the other study.

From a scientific point of view, the FA composition of the liver is of great interest, because endogenous FA synthesis mainly takes place in the liver (Peragón et al., 2000). The dietary treatments (fish oil reduction and supplementation with RV) affected the liver lipid levels and the absolute amounts of EPA and DHA, whereas relative amounts of the FA remained un-changed. A dietary induced change in liver lipid levels is common in salmonids (Caballero et al., 2002; Kjær et al., 2008; Menoyo et al., 2005; Trattner et al., 2008a, 2008b). More interestingly, in this study, the increase in the liver lipid levels led to the increase of absolute amounts of EPA and DHA, which are increased further when dietary RV is given (Table I- 6). RV led to the elevation of DHA in rat hepatocytes (Momchilova et al., 2014), and polyphenol-rich wine lees elevated DHA in zebra fish embryos (Caro et al., 2017). Studies with different model species or cell cultures prove the ability of polyphenols to interact with the hepatic lipid metabolism and FA synthesis. For example, an episesamin/sesamin mixture led to elevated DHA amounts in salmon hepatocytes (Trattner et al., 2008b), and provinol modified the quantity of liver FA in rats (Aoun et al., 2010). In general, the FA compositions of the liver and the whole body

homogenate of rainbow trout did not represent the dietary FA composition, but showed clear influence of RV.

Furthermore, the RV-mediated elevation of DHA in the rat hepatocytes, was ascribed to increased elongase and desaturase activities of Δ5-D and Δ6-D (Momchilova et al., 2014). In accordance with these findings, the Δ6-D protein level significantly increased in the livers of fish fed the F0 + RV diet compared to F0, F4 + RV and F2 + RV fed animals (Figure I- 3). Overall, this indicates that a RV-dependent enhancement of desaturation activity when dietary fish oil content is low, is apparent in the increased abundance of the Δ6-D enzyme. The hepatic Δ6-D protein levels were elevated *in vivo* by dietary RV in the F0 + RV fed fish to a level equal to the untreated controls (diet F4, Figure I-3). The RV-mediated increase of the Δ6-D protein level at 0% dietary fish oil, but decrease of the Δ6-D protein level at 4% dietary fish oil can be explained by a negative feedback. When dietary EPA and DHA is sufficient (in this study 0.74 - 0.91% DM), the further synthesis of EPA and DHA seems to be stopped by RV. Only when the dietary EPA and DHA levels are limited (in this study 0.26 – 0.56% DM), the FA synthesis is enhanced by RV. This indicates the effect of RV on the key enzyme of FA bioconversion that finds expression in elevation of the enzyme itself, as well as EPA and DHA levels in the livers and the whole body homogenate of trout, when dietary n-3 LC-PUFA are insufficient to meet the requirements, and can be regarded as a qualitatively important output.

In contrast, the Δ6-D mRNA expression levels were not increased by RV. The phenomenon of a lack of correlation between Δ6-D mRNA and protein levels was also described by Schiller Vestergren et al. (2012) and Emery et al. (2013). Generally, mRNA expression is very sensitive and rapidly responds to dietary modifications (Schiller Vestergren et al., 2011). Certain phytochemicals, e.g., sesamin and genistein exhibited a time-dependent activation of PPARα and CPT1 mRNA expression *in vitro* (Schiller Vestergren et al., 2011; Trattner et al., 2008b). Thus, in this study, the enhancement of Δ6-D transcription was possibly not detectable in the liver samples taken on the second day after the last feeding. Furthermore, post-transcriptional modifications could also lead to increased mRNA stability (Clarke and Abraham, 1992), resulting in elevated protein levels independent of changes to mRNA levels. The bioavailability of the phytochemical RV was considered to be rather low (Walle et al., 2004) as RV is quickly metabolized, conjugated (by glucuronidation and sulfation (Walle, 2011)), excreted and thus not necessarily available in the tissues over a longer period of time (Delmas et al., 2011). Our results suggest that though there might be low availability of RV, the phytochemical is bioactive and exhibits effects on the fatty acid profile even at low concentrations. Additionally, RV might not only elevate LC-PUFA levels by modifying long chain PUFA conversion on a molecular level, but also through the protection of polyunsaturated fatty

acids from oxidation *via* cell signaling. There is a consistent body of evidence for the antioxidant properties of RV (Frémont et al., 1999; Urquiaga et al., 2004), especially highlighted in a study focusing on the effect of wine polyphenols in human nutrition (Natella et al., 2001). Antioxidant activity could be mediated by the induction of endogenous antioxidant defense mechanisms that are partly under the control of the transcription factor Nrf2 in humans and mice (Wu and Hsieh, 2011). Wang et al. (2015) also recently showed that RV induced the Nrf2 target gene glutathione peroxidase 4 in laboratory rodents, thereby protecting against oxidative stress.

Dietary administration of RV did not significantly modify hepatic PPARα mRNA expression levels (Figure I- 2b). PPARα is a transcription factor which is involved in the regulation of the expression of numerous target genes involved in FA bioconversion and was modified by RV *in vitro* and *in vivo* in mice (Inoue et al., 2003; Tsukamoto et al., 2010). Thus, aside from PPARα mRNA quantification, the mRNA expression levels of selected target genes should also be considered. Administration of RV did not change the hepatic CPT1a and CPT1c mRNA expression. Both CPT1a and CPT1c are located in the mitochondria and involved in regulating the rate of PUFA β-oxidation (Desvergne and Wahli, 1999; Trattner et al., 2008b). The results presented in this study indicated a general regulation of mRNA expression of PPARα, CPT1a and CPT1c by dietary fish oil level regardless of dietary RV. These findings are supported by a study in zebra fish embryos where polyphenol and RV rich wine lees failed to modulate PPAR expression (Caro et al., 2017). Furthermore, findings by Vauzour et al. (2015) indicated that anthocyanidins may not affect FA composition or modify molecular mechanisms of n-3 FA bioconversion. The mechanism of how RV possibly interacts with transcription factors or genes exhibiting a PPAR binding site is yet to be fully understood (Yang et al., 2010). Furthermore, RV presumably interacts *via* mechanisms not investigated in this study. It is well known that RV not only interacts with the FA synthesis, but the hepatic lipid metabolism in general (Baile et al., 2011; Lagouge et al., 2006; Momchilova et al., 2014). Especially the sirtuin mediated pathway in the livers of different model species is greatly affected by RV (for example, mice and humans (Lagouge et al., 2006), and zebra fish (Schirmer et al., 2012)).

Whether RV activates or inhibits distinct signal transduction pathways seems to mainly depend on the dose which is applied in vitro and in vivo. Many phytochemicals and dietary supplements exhibit dose-dependent effects for example, *trans*-ε-viniferin, *cis*-viniferin and gnetin H show dose-dependent cytotoxicity against mouse cancer cell lines *in vitro* (Kim et al., 2002). Additionally, doses of 10 µM genistein, daidzein, and glycitein inhibited estrogen metabolism in primary liver cell culture from Atlantic salmon, lake trout and rainbow trout, whereas lower concentrations had no effect compared to the controls (Ng et al., 2006). Dietary vitamin E supplementation also showed a dose-dependent mode of

action and prevented tissues from lipid oxidation when administered at doses above 100 mg/kg to red sea bream (Gao et al., 2012) and salmon (Faizan et al., 2013). In this study, RV was administered at doses of 3 g/kg, which represented the upper margin of previously applied concentrations in fish (Aluru and Vijayan, 2006; Magrone et al., 2016; Wilson et al., 2015; Yu and Li, 2012). The described effects may help to understand the lack of effects on the expression of certain genes (PPARα, CPT1a and CPT1c), but increased protein abundance of Δ6-D and elevated PUFA contents. Hypothetically, the administered dose of 3 g/kg (RV) might possibly exhibit only minor effects on the level of gene expression, but over the duration of the eight week trial led to increased amounts of the key enzyme in the LC-PUFA synthesis on the protein level. At the same time, protection of LC-PUFAs from oxidation possibly *via* the induction of endogenous antioxidant enzymes elevated their relative amounts.

The main aim of this study was a mechanistic approach to investigate how the phytochemical RV may affect n-3 LC-PUFA synthesis. In rainbow trout, it seems that RV generally shows a great impact on FA synthesis, regardless of the actual bioavailability. It seems to be the case that once RV reaches the liver of rainbow trout, it interacts with the key enzyme of FA synthesis, Δ6-D. Whether this interaction is directly or *via* transcriptional control, cannot be fully explained with the results obtained in this study. No effect of RV on the mRNA expression of Δ6-D, PPARα, CPT1a, and CPT1c could be detected with the methods applied. Nevertheless, the elevated amounts of Δ6-D protein in the livers of fish fed diets with insufficient amounts of EPA and DHA and supplemented with RV are in accordance with increased absolute amounts of hepatic EPA and DHA. Furthermore, also the FA profile of the whole body homogenate of these fish shows elevated amounts of EPA and DHA. Based on literature data and own observations, the processes behind RV mediated activation of the FA synthesis are most likely *via* transcriptional control of Δ6-D and Δ5-D (Figure I- 1) (Caro et al., 2017; Momchilova et al., 2014). The protection of FA from oxidation through RV as a chelator of copper and free-radical scavenger (Frémont et al., 1999) or by interacting with the transcription factor Nrf2 (Wang et al., 2015; Wu and Hsieh, 2011) most likely adds to the effects of RV on Δ6-D. However, it should always be taken into consideration, that RV also interacts with other molecules and pathways (for example uptake of the FA into liver tissue (Delmas et al., 2011), uptake of FA into cell organelles (e.g., acox1 (Tsukamoto et al., 2010)), the interaction with the hepatic sirtuin pathway (Lagouge et al., 2006; Schirmer et al., 2012), or modification of the biochemical pathways affecting adipogenesis in human adipocytes (Baile et al., 2011)) that can have indirect effects on the hepatic FA synthesis pathway. Thus, the observed effects of RV on rainbow trout in this study are the result of many different interactions of RV on a molecular and physiological level, leading to the observed output of elevated EPA and DHA tissue levels.

5. Conclusion

Taken together, the presented data demonstrate an RV-mediated elevation of PUFAs that can putatively be ascribed to an elevation of hepatic Δ6-D protein levels and to the potential antioxidant properties of RV. However, the dietary administration of RV resulted in reduced feed intake in rainbow trout, which may be related to a lower palatability and warrants further research. Nevertheless, RV seems to be a promising dietary supplement that has the ability to increase LC-PUFA levels in farmed rainbow trout fed diets low in fish oil, potentially leading to enhanced fatty acid quality in aquaculture products. Further research should be conducted in other fish species to address the question of what extent RV may affect fatty acid profiles.

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CHAPTER II

Effects of resveratrol and genistein on growth, nutrient utilization, and fatty acid composition of rainbow trout

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Abstract

The replacement of the finite and costly resource fish oil is an important task for aquaculture nutrition. A promising approach could be the use of plant bioactives that may have the potential to influence the metabolism and the synthesis of omega-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA), especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). In this study, the two phytochemicals resveratrol (RV) and genistein (G) were investigated for their effects on fish growth, nutrient utilization, and body nutrient composition alongside their effects on whole body fatty acid (FA) composition. In a feeding trial lasting eight weeks, rainbow trout (initial body weight: 81.4 ± 0.5 g) were held in a recirculating aquaculture system (RAS) and fed six experimental diets with and without fish oil (F4 = 4% of dry matter (DM) and F0 = 0% DM) and supplemented with 0.3% DM of RV and G (feeding groups: F4, F4 + RV, F4 + G, F0, F0 + RV, and F0 + G). The feeding of the F0 + RV diet resulted in reduced feed intake, growth rate, and slightly reduced whole body lipid levels. At the same time, the amount of PUFA and the n-3/n-6 ratio were significantly increased in whole body homogenates of rainbow trout fed diet F0 + RV in comparison to the F0 control. The feeding of the F0 + G diet led to reduced feed intake, slightly increased protein utilization but did not significantly affect the whole body FA composition. Overall, feeding the fish oil-free diet supplemented with the phytochemicals resulted in more pronounced effects on fish performance and FA composition than the single factors *per se* (dietary fish oil level or phytochemical). Present data indicate that G might not be of profitable use for trout nutrition. In terms of FA composition, RV could be a potentially useful complement for fish oil. However, the impairment of growth and performance parameters as observed in the present study discourages its use in trout diets.

Keywords: *Oncorhynchus mykiss*; stilbene; bioactive; LC-PUFA; anti-nutritional factor

1. Introduction

Over the past years, the amount of fish oil used in aquaculture production has decreased mainly due to the increasing demand of fish oil for human consumption as well as increasing market prices (Shepherd and Jackson, 2013). The common practice in aquaculture to manage this imbalance is the substitution of fish oil with vegetable oils in aqua feeds. This practice does not necessarily affect the fish growth and performance, but most likely reduces omega-3 long chain polyunsaturated fatty acid (n-3 LC-PUFA) levels in the end product provided to the consumer (de Roos et al., 2017; Shepherd and Jackson, 2013). Fish and fish derived products, like fish oil, are beneficial for human health in relation to the high amounts of the n-3 LC-PUFAs eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), among other reasons (Molendi-Coste et al., 2011). Thus, a main goal in fish nutrition is to maintain fish n-3 LC-PUFA levels as high as possible to ensure good fish performance, fish health, and a high product quality (Tocher, 2015). An interesting approach in this context is the use of plant derived functional and bioactive phytochemicals which might be potentially useful complements for fish oil replacement in aqua feeds.

In our previous study we have demonstrated that the grape derived stilbene resveratrol (RV) increases the relative n-3 LC-PUFA levels in rainbow trout (*Oncorhynchus mykiss*) most likely *via* an increased production of the hepatic enzyme Δ6-desaturase (Torno et al., 2017). The phytoestrogen genistein (G), a mayor bioactive component in soybean, might affect the lipid metabolism in fish (Cleveland and Manor, 2015) and modulate the expression of genes encoding proteins centrally involved in FA synthesis (Schiller Vestergren et al., 2011). This indicates that G might potentially increase the amount of LC-PUFAs in fish, making it an interesting target phytochemical for fish nutrition studies. However, our previous study indicates that RV might possibly impair fish growth (Torno et al., 2017). Additionally, G is discussed as an anti-nutritional factor (ANF) likely to impair fish growth and performance (Francis et al., 2001). Furthermore, in human nutrition the use of RV and G in obesity and body weight management has been discussed (Rupasinghe et al., 2016). Since RV and G are currently investigated as potential complements for fish oil replacement in aqua feeds further research on their effects on fish growth, performance, and nutrient utilization is indispensable.

Thus, the aim of this study was to evaluate the effects of dietary RV and G in rainbow trout on (1) growth, (2) performance and nutrient utilization parameters, and (3) whole body composition, including the nutrient and fatty acid (FA) composition. We further investigated whether the dietary fish oil level played a role in the response of the investigated parameters.

2. Material and methods

2.1. Experimental diets

Six different experimental diets (isonitrogenous and isoenergetic) were formulated as shown in Table II-1. All diets consisted of mainly alternative plant protein sources and had a fish meal content of 10% dry matter (DM). Diet F4 contained 4 % DM fish oil and 5.93% DM of a mixture of vegetable oils (linseed oil, rapeseed oil and sunflower oil). F4 served as overall reference diet. Diet F0 completely lacked fish oil (0 % DM) and contained 9.93% DM of linseed oil and rapeseed oil. The diets contained considerably different amounts of the fatty acids EPA (20:5n-3), DHA (22:6n-3), and α-linolenic acid (ALA, 18:3n-3) as shown in Table II- 2. Basal diet F4 was formulated to meet the recommended dietary levels of ALA, EPA and DHA for rainbow trout (EPA + DHA: 0.4 - 0.5% of dry diet and ALA: 0.7 - 1.0% of dry diet (NRC, 2011)). Basal diet F0 was formulated to meet ALA requirements, but contained reduced amounts of EPA and DHA below the recommended dietary levels for rainbow trout. Diets F4 + RV and F4 + G were formulated equal to the basal diet F4 but supplemented with resveratrol (+RV; *trans*-3,4',5-trihydroxy stilbene, purity ≥ 98%, CHEMOS GmbH & Co. KG, Regenstauf, Germany) or genistein (+G; 5,7-dihydroxy-3-(4-hydroxyphenyl)-(4H)-benzopyran-4-one, purity ≥ 98%, Chemos GmbH & Co. KG, Regenstauf, Germany)) at 0.3% DM of diet, respectively. The same applied for diets F0 + RV and F0 + G and the basal diet F0, respectively. All diets were formulated based on the macronutrient and the amino acid requirements of rainbow trout according to Rodehutscord et al. (1997) and NRC (2011). All diets were pressed into 4-mm pellets using a feed press L14-175 (Amandus Kahl, Reinbek, Germany).

2.2. Experimental setup

The study was conducted at the facilities of the Gesellschaft für Marine Aquakultur (GMA) mbH in Büsum, Germany. A total of 306 juvenile rainbow trout (Forellenzucht Trostadt GbR, Trostadt, Germany) were allowed to adapt for 1.5 months in a recirculating aquaculture system (RAS). The RAS (7.6 m^3, turnover rate 4 times/h) was equipped with a moving bed bio filter, an additional bead filter (PolyGeyser, Model DF-6, Aquaculture Systems Technologies, L.L.C., New Orleans, USA), and an UV-light disinfection system. The light regime was set at 14:10 h light:dark cycle during the whole adaptation and the experimental period. The water quality parameters were determined daily and maintained in a suitable range for trout (7.54 pH, 18.5 ± 0.7 °C, 7.84 ± 0.3 mg L^{-1} O_2, 84.97 ± 3.22% O_2, 0.3 ± 0.2 mg L^{-1} NH_4, 1.3 ± 0.7 mg L^{-1} NO_2, 2.5 ± 1.1 ‰ PSU). Prior to the experimental start, the acclimated rainbow trout (initial body weight: 81.6 ± 0.5 g) were stocked in six triplicate groups of 17 individuals in 18 tanks (150 L) of the RAS. During the

experimental period lasting eight weeks, the fish were fed manually twice per day until apparent satiation. Uneaten pellets were recollected from the tanks after each feeding for the calculation of the daily feed intake (DFI).

Table II- 1. Ingredients and nutrient composition (percent of dry matter: % DM) of the experimental rainbow trout diets. F4 and F0 are the basal diets, +RV and +G indicate supplementation of basal diets with 0.3% DM resveratrol or genistein, respectively.

Ingredients [% DM]	F4	F4 + G	F4 + RV	F0	F0 + G	F0 + RV
Fish meal (*Clupea sp.*) [1]	10.00	10.00	10.00	10.00	10.00	10.00
Soybean meal [2]	13.50	13.50	13.50	13.50	13.50	13.50
Gelatin [3]	3.00	3.00	3.00	3.00	3.00	3.00
Feather meal [4]	2.00	2.00	2.00	2.00	2.00	2.00
Blood meal [5]	5.50	5.50	5.50	5.50	5.50	5.50
Pea protein isolate [6]	10.00	10.00	10.00	10.00	10.00	10.00
Wheat gluten [7]	23.00	23.00	23.00	23.00	23.00	23.00
Wheat starch [7]	19.50	19.50	19.50	19.50	19.50	19.50
Fish oil [1]	4.00	4.00	4.00	-	-	-
Linseed oil [8]	2.0	2.0	2.0	0.36	0.36	0.36
Rapeseed oil [9]	2.05	2.05	2.05	9.57	9.57	9.57
Sunflower oil [9]	1.88	1.88	1.88	-	-	-
Vitamin mineral premix [10]	0.50	0.50	0.50	0.50	0.50	0.50
Lysine [11]	1.20	1.20	1.20	1.20	1.20	1.20
Calcium hydrogen phosphate [12]	0.50	0.50	0.50	0.50	0.50	0.50
Inert filler [13]	1.37	1.37	1.37	1.37	1.37	1.37
Genistein (G) [14]	-	0.30	-	-	0.30	-
Resveratrol (RV) [14]	-	-	0.30	-	-	0.30
Nutrient composition [% DM]						
Dry matter	84.7	84.2	84.9	82.5	83.9	82.2
Crude protein	52.9	52.7	53.2	53.5	53.1	53.2
Crude lipid	14.7	14.7	14.6	14.9	14.9	14.9
Crude ash	5.7	5.7	5.7	5.7	5.7	5.7
Total carbohydrates [15]	26.7	26.9	26.6	25.9	26.3	26.2
Gross energy [MJ kg $^{-1}$ DM]	22.68	22.68	22.84	22.87	22.82	22.88

[1] Vereinigte Fischmehlwerke Cuxhaven GmbH & Co. KG, Cuxhaven, Germany; [2] SOYAFINE 200, EURODUNA Rohstoffe GmbH, Barmstedt, Germany; [3] Gustav Ehlert GmbH & Co. KG, Verl, Germany; [4] GoldMehl ©FM, GePro Geflügel-Protein Vertriebsgesellschaft mbH & Co. KG, Diepholz, Germany; [5] Blood meal, Sonac, Son, Netherlands; [6] Emsland-Stärke GmbH, Emlichheim, Germany; [7] KRÖNER STÄRKE GmbH, Ibbenbüren, Germany; [8] Makana Produktion und Vertrieb GmbH, Offenbach a.d.Queich, Germany; [9] Different food stores, Büsum, Germany; [10] Emsland-Aller Aqua GmbH, Golßen, Germany; [11] Biolys, Evonik Industries AG, Essen, Germany; [12] J. Rettenmaier und Söhne GmbH und Co. KG, Rosenberg, Deutschland; [13] Bentonite: Del Lago Bentonite, Castiglioni Pes y Cía., Buenos Aires, Argentina; [14] CHEMOS GmbH & Co. KG, Regenstauf, Germany; [15] Total carbohydrates = 1000 - (crude protein + crude fat + crude ash)

Table II- 2. Fatty acid composition (percentage of total fatty acid methyl esters (% of FAMEs) and % of dry matter (DM) of diet) of the experimental rainbow trout diets. F4 and F0 are the basal diets with 4% DM and 0% DM fish oil, +RV and +G indicate supplementation of basal diets with 0.3% DM resveratrol or genistein, respectively

[% of FAMEs]	F4	F4 + G	F4 + RV	F0	F0 + G	F0 + RV
14:0	1.81	1.88	1.88	0.43	0.38	0.36
16:0	12.38	12.35	12.29	8.88	8.64	8.57
18:0	3.94	3.82	3.76	3.00	2.89	2.90
Σ SFA [1]	18.13	18.06	17.93	12.31	11.91	11.83
14:1	0.05	0.04	0.04	0.01	0.01	0.01
16:1	1.70	1.69	1.68	0.53	0.49	0.48
18:1n-9c	29.98	29.53	29.58	48.16	48.72	49.02
18:1n-7c	2.14	2.12	2.14	3.07	3.04	2.96
Σ MUFA [2]	33.86	33.38	33.44	51.78	52.27	52.47
18:2n-6	30.11	30.59	30.63	25.82	25.65	25.57
18:3n-6	0.06	0.05	0.05	0.01	0.26	0.26
18:3n-3 [3]	11.97	12.18	12.22	8.65	8.61	8.63
20:5n-3 [4]	2.14	2.19	2.18	0.47	0.42	0.41
22:5n-3	0.54	0.54	0.54	0.17	0.16	0.15
22:6n-3 [5]	3.19	3.01	3.01	0.79	0.73	0.67
Σ PUFA [6]	48.01	48.56	48.63	35.92	35.83	35.70
20:5n-3 + 22:6n-3	5.33	5.20	5.19	1.27	1.15	1.08
20:5n-3 / 22:6n-3	0.67	0.73	0.72	0.59	0.59	0.61
18:3n-3 / 18:2n-6	0.40	0.40	0.40	0.33	0.34	0.34
18:3n-3 % DM [7]	1.63	1.67	1.65	1.20	1.20	1.20
20:5n-3 % DM [7]	0.29	0.29	0.30	0.07	0.06	0.06
22:6n-3 % DM [7]	0.44	0.41	0.41	0.11	0.09	0.10
20:5n-3 + 22:6n-3 % DM [7]	0.73	0.70	0.71	0.18	0.15	0.16

[1] ∑ SFA is the sum of saturated fatty acids; [2] ∑ MUFA is the sum of monounsaturated fatty acids; [3] 18:3n-3: α-Linolenic acid (ALA); [4] 20:5n-3: Eicosapentaenoic acid (EPA); [5] 22:6n-3: Docosahexaenoic acid (DHA); [6] ∑ PUFA is the sum of n-3 and n-6 polyunsaturated fatty acids; [7] Fatty acids in % DM of diet were calculated based on 13:0 methyl ester as the internal standard and amount of lipid in the diet (Table II- 1).

2.3. Sampling

Tissue samples (blood, liver, whole body) were collected before the onset (day 0, initial) and at the end of the feeding trial (day 62). At day 0, five individuals were sacrificed (pooled sample) and stored at −20 °C for the determination of the whole body nutrient composition and the whole body fatty acid composition. At day 62, three individuals per tank were sacrificed (pooled samples) and stored at −20 °C for the same analysis. Blood

was collected for the determination of the hematocrit (HK) using micro hematocrit tubes (diameter = 1.15 ± 0.05 mm, Brand GmbH + Co KG. Wertheim, Germany). HK was determined in a triple approach per individual and five individuals per tank. Individual weight (± 0.01 g), length (± 0.1 cm), and liver weight (± 0.0001 g) were determined from five individuals per tank for the calculation of the Fulton condition factor (FCF) and the Hepatosomatic index (HSI). Based on an additional group weighing of all individuals from each tank, growth, performance, and nutrient utilization parameters (specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and protein productive value (PPV)) were calculated (see formulas in Table II-3).

2.4. Nutrient composition analysis

The nutrient composition was determined in all experimental diets and the whole body homogenates of the rainbow trout. First, frozen whole body samples were freeze-dried (Alpha 1-2 LDplus and Alpha 1-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until the weight was stable. All samples were homogenized using a cutting mill (GM 200, Retsch, Haan, Germany) or mortar and pestle and stored at 4 °C. Analysis of dry matter (DM), crude ash, crude protein, crude lipid, total carbohydrates, and gross energy was done according to EU guideline (EC) 152/2009 (European Union, 2009). For the determination of DM, the homogenized samples were dried at 103 °C in a drying oven until the weight remained stable. Ash content was determined after 4 h incineration at 550 °C in a combustion oven (P300, Nabertherm, Lilienthal, Germany). The Kjeldahl method (InKjel 1225M, WD30, Behr, Düsseldorf, Germany) was used for the determination of the crude protein content (nitrogen x 6.25). The crude lipid content was quantified following extraction with petroleum ether in a Soxleth extraction system (Soxtherm, Hydrotherm, Gerhardt, Königswinter, Germany). The diet samples were hydrolyzed with hydrochloric acid prior to the lipid extraction. Gross energy was measured using a bomb calorimeter (C 200, IKA, Staufen, Germany). Total carbohydrates were calculated according to the formula in Table II- 1.

2.5. Lipid extraction and fatty acid measurement

The extraction of total lipids from the whole body homogenates and the diet samples was done according to Folch et al. (1957) and the measurement of fatty acid methyl esters (FAMEs) was performed using a gas chromatograph with flame ionization detector (GC-FID; Agilent Technologies, Santa Clara, CA, USA). In brief, methylation of fatty acids and extraction of methylated FAMEs was conducted with the help of the Folch reagent (chloroform:methanol 2:1). Samples were neutralized using potassium hydroxide (0.1 M) and FAMEs were isolated by the addition of the Folch reagent and subsequent centrifugation for 10 min at 2000× *g*. The organic phase was collected and a second

extraction with potassium hydroxide and the Folch reagent was performed, followed by centrifugation (5 min at 2000× *g*) and drying of samples under a N_2 flux. Re-dissolved FAME samples were injected into a 7820A Agilent gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent HP-88 fused silica capillary column (60 m × 250 µm × 0.2 µm, Agilent Technologies) and helium (1.2 mL/min) as the carrier gas. The following temperature protocol was applied: initial temperature 125 °C, ramp 8 °C/min to 145 °C (26 min), ramp 2 °C/min to 220 °C (5 min). Chromatograms were recorded and analyzed using EZChrom Elite software (Agilent Technologies). A FAME standard (13 FAMEs, see Table II- 2) was used to identify the retention times of the individual FAMEs. Fatty acid composition was calculated as a percentage of single FAME relative to total FAMEs. FAs as % DM of diet were calculated using 13:0 methyl ester as the internal standard.

2.6. Statistical analysis

All statistical Analysis was performed using R (version 3.1.3) with an RStudio interface. The packages *gdata, multcomp, gplots, nlme, SimComp*, and *car* were used for the graphical and the statistical analysis. The data evaluation started with the definition of an appropriate statistical model based on a graphical residual analysis of the data: (1) statistical model based on generalized least squares (*gls*) for normally distributed and heteroscedastic data (FBW, FCR, SGR, PER and PPV); (2) linear model (*lm*) for normally distributed and homoscedastic data (DFI, IBW, whole body composition and fatty acid composition); (3) mixed model (*lme*) for non-normally distributed data (FCF, HSI and HK). All models included the level of dietary fish oil content (4 % and 0 %) and phytochemical (None, RV, G), as well as their interaction term and *lme* additionally included tank as random factor. An analysis of variances (ANOVA) was conducted followed by an appropriate post-hoc test: (1) ANOVA based on *gls* was followed by multiple contrast tests for heteroscedastic data according to Hasler & Hothorn (2008); (2) ANOVA based on *lm* was followed by multiple contrast tests (Schaarschmidt and Vaas, 2009); (3) ANOVA based on *lme* was followed by multiple contrast tests for relative effects (Schaarschmidt and Vaas, 2009).

3. Results

3.1. Resveratrol and genistein affected growth, performance, and nutrient utilization

The overall growth of the fish was rather poor (between 41% and 69% weight increase) over the eight weeks feeding trial with SGR under 0.85 % d^{-1} and FCR above 2.6 (both F0 + G; Table II- 3).

Table II- 3. Growth, performance, and nutrient utilization parameters of rainbow trout fed with the experimental diets for eight weeks. F4 and F0 indicate feeding of the basal diets (4% DM) and 0% DM fish oil). +RV and +G indicate feeding groups that were fed diets supplemented with resveratrol or genistein (0.3% DM), respectively.

	F4	F4 + G	F4 + RV	F0	F0 + G	F0 + RV	RSE [§]	Standard deviation (tank)
IBW [1]	81.3	81.8	81.5	81.6	81.1	81.1	0.353	-
FBW [2]	124.9 (14.3)	123.3 (19.5)	135.2 (22.9)	127.9 (9.6) [ab]	137.3 (4.5) [a]	114.7 (3.4) [b]	-	-
SGR [3]	0.69 (0.19)	0.65 (0.25)	0.80 (0.28)	0.72 (0.12) [ab]	0.85 (0.06) [a]	0.56 (0.04) [b]	-	-
DFI [4]	2.55 [x]	2.17 [y]	2.32 [xy]	2.70 [a]	2.21 [b]	2.21 [b]	0.147	-
FCR [5]	4.01 (1.65)	3.67 (1.29)	3.27 (1.61)	3.79 (0.42)	2.60 (0.12) [(b)]	3.98 (0.44) [(a)]	-	-
PER [6]	0.6 (0.2)	0.7 (0.3)	0.8 (0.3)	0.6 (0.1) [b]	0.9 (0.0) [a]	0.6 (0.1) [b]	-	-
PPV [7]	9.3 (2.8)	10.9 (4.3)	12.3 (5.0)	9.7 (1.0) [b]	13.9 (0.2) [a]	9.5 (1.3) [b]	-	-
HSI [8]	1.13 (0.15) [x]	1.05 (0.16) [xy]	0.99 (0.13) [y]	1.04 (0.14)	1.12 (0.12)	1.04 (0.18)	-	0.062
FCF [9]	1.22 (0.16)	1.15 (0.18)	1.22 (0.17)	1.21 (0.25)	1.24 (0.09)	1.14 (0.24)	-	0.066
HK [10]	32.1 (6.6)	32.2 (4.8)	34.6 (4.2)	32.7 (6.9)	34.8 (5.1)	33.5 (4.5)	-	2.727

[1] IBW = Initial body weight [g]; [2] FBW = Final body weight [g]; [3] SGR = Specific growth rate [% d^{-1}] = [ln (FBW) − ln (IBW)] / feeding day × 100; [4] DFI = Daily feed intake [% d^{-1}]; [5] FCR = Feed conversion ratio = feed intake [g] / weight gain [g]; [6] PER = Protein efficiency ratio = weight gain [g] / protein intake [g]; [7] PPV = Protein productive value [%] = 100 × [(final body protein × final body weight) − (initial body protein x initial body weight)] / (protein intake); [8] HSI = Hepatosomatic index [%] = (liver weight / final body weight) × 100; [9] FCF = Fulton condition factor = 100 × (final body weight × final body length^{-3}); [10] HK = Hematocrit [%]; [§] RSE = Residual standard error

Mean values (n=3; HSI and FCF: n=15, HK: n=12) with different superscript letters within one row significantly differ based on ANOVA followed by post-hoc tests (p-values < 0.05) as described in Materials & methods. Superscript letters indicate the output based on comparisons of phytochemical type within one fish oil inclusion group (a, b: F0 diets; x, y: F4 diets). Superscript letters in brackets indicate a tendency towards a statistical difference based on a p-value < 0.1. The RSE are given based on the statistical model used for each parameter: group-RSE (in brackets behind each group) when a statistical model based on generalized least squares (*gls*) was used; RSE for all groups together when a linear model (*lm*) was used; Standard deviation explaining the tank variation additionally to the group-RSE when a mixed model (*lme*) with tank as random factor was used (see Material & methods).

Fish fed the diets with 0% DM fish oil exhibited the strongest response to dietary RV and G as far as growth, performance, and nutrient utilization parameters are concerned (Table II- 3). The FBW and SGR were significantly higher in fish fed the F0 + G diet in comparison to F0 + RV ($p < 0.05$, Table II- 3, indicated by letters a, b), but not in comparison to the F0 fed controls. The PER and PPV were generally low (PER < 0.9 and PPV < 13.9%; both F0 + G), but significantly elevated in the fish fed the F0 + G diet compared to fish that received the F0 and F0 + RV diets ($p < 0.05$, Table II- 3, indicated by letters a, b). Throughout the whole experimental period, RV and G administration significantly lowered the DFI in comparison to ingestion of both the basal diet F4 and F0, respectively. The HSI was significantly reduced in the F4 + RV fed group in comparison to the F4 fed control ($p < 0.05$, Table II- 3, indicated by letters x, y). The FCF and the HK remained largely unaffected by the dietary treatments.

The evaluation of the statistical significance of effects revealed that the effects on FBW, SGR, DFI, FCR, PER, and PPV, though more pronounced in fish fed F0-based diets, can be primarily assigned to the dietary phytochemicals RV and G. The dietary fish oil level *per se* did not alter the outcome of the abovementioned parameters (Table II- 4).

Table II- 4. P-values from the ANOVAs conducted on the effects of different experimental diets on selected performance parameters and fatty acid composition of rainbow trout at the end of the eight week feeding trial. The table shows the statistical significance of effects caused by dietary fish oil level, dietary phytochemical supplementation, and the interaction of both (dietary fish oil level and phytochemical supplementation).

	Fish oil	*Phytochemical*	*Interaction*
FBW [1]	0.250	<0.0001 ***	0.186
SGR [2]	0.201	<0.0001 ***	0.176
DFI [3]	0.683	<0.0001 ***	0.348
FCR [4]	0.182	0.0001 ***	0.377
PER [5]	0.287	<0.0001 ***	0.325
PPV [6]	0.251	<0.0001 ***	0.348
MUFA [7]	0.078 (*)	0.135	0.038 *
EPA	0.107	0.602	0.056 (*)
DHA	0.078 (*)	0.229	0.064 (*)
PUFA [8]	0.327	0.564	0.008 **
EPA+DHA	0.070 (*)	0.217	0.029 *
EPA/DHA	0.390	0.062 (*)	0.474
n-3/n-6 [9]	0.076 (*)	0.509	0.107

[1] FBW = Final body weight; [2] SGR = Specific growth rate; [3] DFI = Daily feed intake; [4] FCR = Feed conversion ratio; [5] PER = Protein efficiency; [6] PPV = Protein productive value; [7] MUFA is the sum of all monounsaturated fatty acids; [8] PUFA is the sum of n-3 and n-6 polyunsaturated fatty acids; [9] n-3/n-6 is the ratio between all n-3 fatty acids and all n-6 fatty acids measured

Statistical significant differences are indicated as *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$), and (*) for a tendency towards statistical difference based on a p-value < 0.1.

3.2. Dietary treatment only slightly affected the body composition

The whole body composition of the rainbow trout marginally differed between the dietary treatments at the end of the feeding trial (Table II- 5). Crude ash was significantly elevated in fish fed the F0 + G and F0 + RV diets compared to their F0 fed counterparts ($p < 0.05$, Table II- 5, indicated by letters a, b). Furthermore, trout fed the F0 + RV diet displayed a slightly increased crude ash content in comparison to the F4 + RV fed group ($p < 0.05$, Table II- 5, indicated by letters A, B). Crude lipid slightly tended to a reduction in the F0 + RV group compared to the F0 group ($p < 0.1$, Table II- 5, indicated by (a), (b)). All other parameters investigated remained unaffected by the dietary treatments.

Table II- 5. Body composition (percentage of original matter (% OM) or MJ kg $^{-1}$ OM) of whole body homogenates of rainbow trout at the beginning (initial) and the end of an eight week feeding trial. F4 and F0 represent results that were obtained from fish fed the basal diets containing 4% and 0% DM fish oil. Supplementation of diets with resveratrol and genistein is indicated by +RV and +G (0.3% DM), respectively.

Body composition [% OM]	Initial	F4	F4 + G	F4 + RV	F0	F0 + G	F0 + RV	RSE [1]
Dry matter	28.3	22.6	22.4	22.9	23.2	22.1	21.7	0.905
Crude protein	16.1	15.8	16.1	16.1	16.1	16.1	16.2	0.217
Crude lipid	9.1	3.6	3.1	3.6	4.0 $^{(a)}$	2.7	2.2 $^{(b)}$	0.729
Crude ash	2.8	3.2	3.2	3.2 ^{B}	3.1 ^{b}	3.3 ^{a}	3.3 ^{aA}	0.076
Gross energy [MJ kg $^{-1}$ OM]	7.59	5.20	5.03	5.24	5.37	4.88	4.71	0.331

[1] RSE = Residuals standard error

Mean values (n=3, Initial n=1) and RSE based on a linear model (lm) and ANOVA as described in Materials & methods. Superscript letters indicate the output of the post-hoc tests (p-values < 0.05) based on comparisons of phytochemical type within one fish oil inclusion group (a, b: F0 diets) or fish oil level within one phytochemical supplementation group (A, B: +RV diets). Brackets indicate a tendency towards a statistical difference based on a p-value < 0.1.

3.3. Dietary treatment affected whole body fatty acid composition

The FA composition of the experimental diets was equally altered in the basal diets (F4 and F0) and in the corresponding supplemented diets (+RV and +G, Table II- 2). The amounts of saturated FA (SFA) and PUFA were reduced in the diets with 0% DM fish oil (F0, F0 + G, and F0 + RV), whereas the amount of monounsaturated FA (MUFA) was increased. Most importantly, the amounts of EPA + DHA were reduced from approximately 0.72% DM (F4 diets) to approximately 0.16% DM (F0 diets, Table II- 2). The FA compositions of the whole body homogenates of rainbow trout were neither significantly modified by altering the dietary fish oil level (group F4 in comparison to

group F0) nor by dietary administration of G (Table II- 6). The relative amounts of EPA, DHA, EPA + DHA, and PUFA significantly increased in fish fed the diet F0 + RV in comparison to F4 + RV, whereas the amounts of MUFA decreased ($p < 0.05$, Table II-6, indicated by letters A, B). Thus, in the RV-treated individuals, the tissue EPA + DHA levels were significantly increased from 7.52% FAMEs (F4 + RV) to 10.97% FAMEs (F0 + RV, $p < 0.05$, Table II- 6, indicated by letters A, B). Hence, the ratio of n-3/n-6 LC-PUFA was significantly elevated in fish fed the F0 + RV diet in comparison to the F4 + RV fed fish ($p < 0.05$, Table II- 6, indicated by letters A, B).

Moreover, RV supplementation to the F0 diet (F0 + RV) significantly affected the FA composition of rainbow trout in comparison to sole F0 feeding. The amount of MUFA was significantly reduced in fish fed diet F0 + RV than in fish fed diet F0 ($p < 0.05$, Table II- 6, indicated by letters a, b). The relative amounts of PUFA were significantly higher in fish fed the F0 + RV diet (32.19% FAMEs) than in fish fed the F0 diet (29.03% FAMEs, $p < 0.05$, Table II- 6, indicated by letters a, b). Additionally, the amounts of EPA, DHA, and EPA + DHA tendentially increased in the fish fed the F0 + RV diet in comparison to F0 fed fish ($p < 0.1$, Table II- 6). The relative levels of PUFA and EPA + DHA were significantly affected by the interaction of the dietary fish oil level and RV supplementation and not the single factors *per se*, as proven by the evaluation of the statistical significance of effects (Table II- 4).

4. Discussion

This study revealed that the DFI was significantly impaired by both, dietary RV and G, which presumably led to the impairment of other parameters. Possibly, the acceptance of supplemented diets was reduced by the sensory impact of the phytochemicals, which has been described for feed additives in various fish species (Francis et al., 2001). It is well known, that RV and G may act as ANFs by reducing the appetite or affecting the taste of the feed (bitterness and astringency), thereby resulting in lower feed and thus energy intake (Kim et al., 2006; Nakata et al., 2012). Though the feed intake in G-fed fish was reduced, the utilization of protein (PER and PPV) was significantly enhanced, leading to growth rates comparable to the control-fed groups (Table II- 3). Estrogen-like secondary plant compounds can affect growth and other physiological parameters in fish depending on the species or developmental stage investigated (Cleveland and Manor, 2015; Pastore et al., 2018). Possibly, the estrogenic properties of G might have caused the slight alterations in nutrient utilization and growth as observed within this study. Depending on the dose administered, RV and G might exhibit either beneficial or detrimental bioactivity in animals (Francis et al., 2001; Mukherjee et al., 2010).

Table II- 6. Fatty acid composition (percentage of total fatty acid methyl esters (% FAMEs)) of whole body homogenates of rainbow trout before (initial) and at the end of the feeding trial. F4 and F0 represent results that were obtained from fish fed the basal diets containing 4% and 0% DM fish oil. Supplementation of diets with resveratrol and genistein is indicated by +RV and +G (0.3% DM), respectively.

[% FAMEs]	Initial	F4	F4 + G	F4 + RV	F0	F0 + G	F0 + RV	RSE [§]
SFA [1]	16.47	17.08	17.83	18.70	17.58	19.20	19.09	1.059
MUFA [2]	54.61	52.12	51.50	53.25 [A]	53.39 [a]	50.12 [ab]	48.71 [B,b]	1.707
PUFA [3]	28.92	30.80	30.67	28.67 [B]	29.03 [b]	30.84 [ab]	32.19 [A,a]	1.221
EPA [4]	1.13	1.19	1.13	0.99 [B]	1.07	1.29	1.31 [A]	0.141
DHA [5]	5.67	7.33	7.62	6.53 [B]	6.59 [(b)]	8.85	9.67 [A(a)]	1.273
EPA+DHA	6.8	8.53	8.75	7.52 [B]	7.66 [(b)]	10.14	10.97 [A(a)]	1.399
EPA/DHA	0.2	0.16	0.15	0.15	0.16 [(a)]	0.15	0.14 [(b)]	0.013
n-3/n-6 [6]	0.72	0.76	0.78	0.70 [B]	0.72	0.89	0.91 [A]	0.157

[1] SFA is the sum of saturated fatty acids; [2] MUFA is the sum of monounsaturated fatty acids; [3] PUFA is the sum of n-3 and n-6 polyunsaturated fatty acids; [4] EPA: Eicosapentaenoic acid, 20:5n-3; [5] DHA: Docosahexaenoic acid, 22:6n-3; [6] n-3/n-6 is the ratio between all n-3 fatty acids and all n-6 fatty acids measured, [§] RSE = Residual standard error

Mean values (n=3, Initial n=1) and RSE based on a linear model (lm) and ANOVA as described in Materials & methods. Superscript letters indicate the output of post-hoc tests (p-values < 0.05) based on comparisons of phytochemical type within one fish oil inclusion group (a, b: F0 diets) or dietary fish oil level within one phytochemical supplementation group (A, B: +RV diets). Brackets indicate a tendency towards a statistical difference based on a p-value < 0.1.

Results from our study indicate, that the use of 0.3% DM of G has no significant beneficial effect on the fish growth and performance. In contrast, 0.3% DM of RV led to impaired growth parameters under the given experimental conditions. The use of the pure phytochemical RV in the fish oil free diet F0 + RV revealed negative effects on the growth performance (FBW and SGR) and nutrient utilization (FCR) in comparison to the F0 fed control fish.

Different mechanisms of how RV possibly reduces the nutrient utilization and the growth rate are currently discussed (Rupasinghe et al., 2016). Polyphenols might form polyphenol-protein-complexes that shield the proteins from digestion (Stojadinovic et al., 2013) and thus may impair nutrient availability and growth. Furthermore, polyphenols have been shown to inhibit the activity of proteases, pepsin and trypsin, and lipases (McDougall et al., 2008; Ozdal et al., 2013; Stojadinovic et al., 2013), thereby reducing the digestibility of macronutrients. In this study, RV-treated fish had a slightly lower body lipid level compared to controls (Table II- 5), which might indicate an interaction of RV with the lipid metabolism of rainbow trout. An interaction of RV with the sirtuin pathway and the AMP-activated protein kinase (AMPK) seems likely (Ajmo et al., 2008; Zang et al., 2006). Furthermore, RV-rich wine lees lead to a reduction of lipid content in zebra fish (*Danio rerio*) embryos (Caro et al., 2017). Own investigations revealed, that RV and G both potentially reduce the digestibility of crude lipid and gross energy (Torno et al., 2018) and thus might have led to the reduced lipid content and impaired growth rates of the experimental fish in the current study. Though exact underlying cellular and molecular mechanisms were beyond the scope of this study, a slight lipid-lowering effect of dietary RV can solely be assumed.

The reduction of dietary fish oil from 4% DM to 0% DM did not affect the growth and performance parameters of rainbow trout, which is in accordance with the common and commercially applied practice of fish oil replacement (Lazzarotto et al., 2015). Nevertheless, the overall growth, performance, and nutrient utilization of the fish were rather poor in this study (Table II- 3). The DFI in this study was adequate (> 2.21 % d^{-1}), but in combination with poor FCR (> 2.6) led to low SGR below estimations (> 3 % d^{-1} by Austreng et al. (1987)). Furthermore, PER and PPV values were rather low and did not reach values as reported in other studies (PER > 1.04 (Steffens, 1981)) or indicated by the National Research Council (PPV approximately 31% (NRC, 2011)). An impairment of growth, FCR, and protein utilization caused by diets rich in plant derived components has been previously observed (Drew et al., 2007). We assume that especially the poor performance of the control fed fish indicated an additional systemic effect, which led us to an early termination of the experiment. From experience with bioactive compounds previously used in the RAS from our facility we may assume that a possible accumulation

of RV and G in the holding water might have affected the overall performance of fish in the RAS. Both phytochemicals have a rather low bioavailability and might thus be excreted into the holding water in significant amounts (Gontier-Latonnelle et al., 2007; Walle et al., 2004).

In this study, the EPA + DHA levels in the diets were reduced from 0.72% DM (F4) to 0.16% DM (F0), which is below the suggested amount of 0.4 – 0.5% DM (NRC, 2011). Simultaneously, the total n-3 LC-PUFA levels in the fish were similar between F4 and F0 fed animals, thus indicating the previously described active *in vivo* LC-PUFA metabolism in trout (Gregory et al., 2016) when the dietary supply of EPA and DHA is insufficient. Furthermore, dietary resveratrol led to a decrease of MUFA and an increase of PUFA and EPA + DHA content in the whole body of the fish. The amounts of EPA + DHA were increased by approximately 29% and 19% (F0 + RV and F0 + G, respectively) in comparison to the F4 fed control (Table II- 6). When the phytochemicals were supplemented to diets containing 4% DM fish oil no difference in FA composition was observed. Thus, the increase in LC-PUFA levels was not solely caused by the phytochemical *per se*, but the interaction of the elimination of dietary fish oil and simultaneous administration of the phytochemical (Table II- 4). A similar effect was observed in our previous study, where RV only enhanced the hepatic enzyme production of the Δ6-desaturase, a key enzyme in LC-PUFA synthesis, when trout were fed diets lacking fish oil (Torno et al., 2017). A bi-directional interaction of RV with the lipid metabolism of zebra fish has been recently investigated (Ran et al., 2017). The n-3 LC-PUFA enhancing effects of RV could be additionally mediated through a direct impact on other elongases and desaturases in the FA synthesis (Momchilova et al., 2014). This mechanism might only be of importance when the endogenous FA synthesis is enhanced in the case of insufficient dietary EPA and DHA supply (Gregory et al., 2016). The results obtained in this study indicate, that RV might be used as a complement for fish oil replacement in terms of modulation of the FA composition of rainbow trout. Furthermore, we were not able to identify significant LC-PUFA enhancing properties of G in rainbow trout.

The assessment of the statistical significance of the effects observed in this study revealed that the phytochemicals significantly affected growth, performance, and nutrient utilization (Table II- 4). The FA composition of rainbow trout was modified by the interaction of the phytochemical RV and the dietary fish oil level. It has been reported that rainbow trout can compensate for the lacking dietary fish oil and lacking dietary n-3 LC-PUFA in a way that the performance remains unaffected (Lazzarotto et al., 2015). In this study, effects of RV on fish growth and performance were only present, when the fish diet completely lacked EPA and DHA (F0 diets). This indicates, that the fish oil free

diet in combination with the applied concentration of RV challenged the trout beyond their ability to cope with one of both factors.

5. Conclusion

In conclusion, dietary G exhibited only a slight capability, if any, to affect the growth, performance, and n-3 LC-PUFA levels, indicating lacking profitable use in rainbow trout production. Dietary RV led to a significant increase of PUFA and n-3/n-6 ratio in rainbow trout tissue, but adversely affected the growth and the nutrient utilization. A general impairment of the overall fish performance as observed in this study suggests careful considerations regarding the used concentration as well as the potential accumulation of phytochemicals in recirculating systems. The impairment of growth and production parameters overweighs the positive effects of RV and questions its successful application in rainbow trout diets.

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CHAPTER III

Effects of resveratrol and genistein on nutrient digestibility and intestinal histopathology of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Current trends in aquaculture production have led to the interesting approach of using plant-derived bioactives and phytochemicals in fish nutrition. The potential use of phytochemicals covers, among many other aspects, also the search for substances that increase the nutritional value of aquaculture products. Apart from the targeted beneficial effects, dietary supplements might exert adverse side effects. In this study, resveratrol and genistein were investigated for their effects on nutrient digestibility and intestinal histopathology of rainbow trout. A three-week digestibility trial was conducted with rainbow trout (144.2 ± 16.1 g) fed three experimental diets: diet CD was the control diet, diet RV was supplemented with resveratrol (0.3% dry matter of the diet), and diet G was supplemented with genistein (0.3% dry matter of the diet). The trial revealed slightly negative effects of the phytochemical-supplemented diets on the digestibility of dry matter (diet G), crude lipid (diet G), and gross energy (diets G and RV). The histopathological investigation of the hindgut revealed neither damage nor improvement of the intestinal morphology through resveratrol and genistein. Thus, microscopic changes and inflammation could be excluded as reason for the reduced digestibility. Overall, the effects of resveratrol and genistein on the digestibility of macronutrients are considered as one possible mechanism impairing growth and performance of rainbow trout.

Keywords: stilbene; phytoestrogen; antinutrient; apparent digestibility coefficient; morphology

1. Introduction

Phytochemicals and plant-derived supplements are increasingly investigated for their use in diets of aquaculture fish. Plant-derived substances may function as immuno-stimulants, anti-pathogenic, and anti-stress agents (Bulfon et al., 2015; Chakraborty and Hancz, 2011; Holm et al., 2016). Furthermore, the intensification of aquaculture and growing demand for fish reinforced the search for supplements that improve fish growth rates and performance (Immanuel et al., 2009; Ji et al., 2007a, 2007b). Another approach is the application of plant bioactives that potentially affect certain nutritive aspects of aquaculture products (Villasante et al., 2015; Welker et al., 2016). Especially in finfish and salmonid aquaculture, the use of phytochemicals that improve the fatty acid composition in favor of n-3 long chain polyunsaturated fatty acids seems to be a promising strategy (Faizan et al., 2013; Kühn et al., 2016; Schiller Vestergren et al., 2012; Torno et al., 2017; Trattner et al., 2008). In our previous study, we investigated the effects of the phytochemical resveratrol on the growth and the fatty acid composition of rainbow trout (*Oncorhynchus mykiss*) (Torno et al., 2017). The study revealed that resveratrol putatively exhibits detrimental side effects on fish growth, most likely caused by reduced feed intake. Furthermore, a potential impairment of feed utilization and digestibility of nutrients caused by the phytochemical has not yet been investigated. Resveratrol is a polyphenol and stilbene derivate of grape vines and is produced by the plant in response to infections (Gresele et al., 2011). When fed to animals, polyphenols may reduce the availability (Ozdal et al., 2013) and digestibility (Stojadinovic et al., 2013) of proteins and other nutrients.

Another interesting bioactive phytochemical investigated by our group and already discussed in fish nutrition for its potential beneficial but also detrimental effects is genistein (Cleveland and Manor, 2015; Schiller Vestergren et al., 2011). Genistein is an isoflavonoid present in soybean that possesses estrogenic as well as antimicrobial activity (Dixon and Ferreira, 2002) and might impair fish growth (Cleveland and Manor, 2015; DiMaggio et al., 2016; Mambrini et al., 1999; Romarheim et al., 2008). Genistein is further discussed as one of the anti-nutritional factors (ANFs) responsible for gastrointestinal inflammation and soybean-induced enteritis, thus reducing the digestibility of macronutrients (Baeverfjord and Krogdahl, 1996; Francis et al., 2001; van den Ingh et al., 1996).

If both phytochemicals are potentially to be used in aquafeeds for their beneficial effects, it is essential to investigate their adverse side effects. Thus, the aim of the current study was to elucidate the effects of dietary resveratrol and genistein on the digestibility of macronutrients and histopathological changes in the intestine of rainbow trout fed predominantly vegetable diets.

2. Materials and methods

2.1. Experimental setup

2.1.1. Experimental diets

Three experimental diets (isonitrogenous and isoenergetic) were formulated as shown in Table III-1. All diets contained 1% DM titanium dioxide (TiO_2) as inert marker for the determination of apparent digestibility coefficients (ADCs). Diet CD served as the control diet. The other two diets were equal to diet CD but supplemented with 0.3% DM resveratrol (diet +RV) or 0.3% DM genistein (diet +G), respectively. The amino acid (AA) contents of the diets were calculated based on AA contents of the single ingredients. All diets were formulated to fit the nutrient and AA requirements of rainbow trout according to Rodehutscord et al. (1997) and NRC (2011). Pelleted diets were prepared using a feed press L14-175 (Amandus Kahl, Reinbek, Germany) equipped with a 4 mm matrix.

2.1.2. Experimental setup

The trial was conducted at the facilities of the Gesellschaft für Marine Aquakultur (GMA) mbH in Büsum, Germany. A total of 300 juvenile rainbow trout (Forellenzucht Trostadt GbR, Trostadt, Germany) were allowed to acclimatize for 2 weeks in a recirculating aquaculture system (RAS). The experimental RAS (20 m^3, turnover rate 2.4 times h^{-1}) was equipped with a moving bed bio filter, a drum filter (20 µm mesh size), oxygen cone, protein skimmer, ozone sterilizer, and an UV-light disinfection system. The light regime was set at 15:9 h light:dark cycle during the whole acclimatization and the experimental period. The water quality parameters were determined daily and maintained in a suitable range for rainbow trout (7.14 pH, 15.7 ± 0.6 °C, 90.2 ± 5.1% O_2, 0.2 ± 0.2 mg L^{-1} NH_4, 0.3 ± 0.2 mg L^{-1} NO_2 (Microquant test kit for NH_4 and NO_2 Merck, Germany), 5.9 ± 0.8 ‰ PSU salinity).

Rainbow trout (initial body weight: 144.2 ± 16.1 g) were randomly sorted into nine groups consisting of 30 individuals each and branded using differently colored visible implant elastomer (VIE) tags (NMT – Northwest Marine Technology, Inc., Shaw Island, WA, USA). To allow triplicate measurements, each holding tank (600 L) was stocked with fish from three differently branded groups (n = 3 per tank). Each tank represented one of the three feeding groups CD, +RV, and +G. During the whole experimental period of three weeks, the fish were manually fed 1.4% of their body weight daily in one feeding event per day. The ration was set at 1.4% of the body weight to ensure that no uneaten pellets remained in the tank and all feeding groups were fed equal rations.

Table III- 1. Ingredients and nutrient composition (percent of dry matter (% DM)) of the experimental diets. CD is the control diet, +RV the diet containing resveratrol, and +G the diet containing genistein.

Ingredients [% DM]	CD	+RV	+G
Fish meal (*Clupea sp.*) [a]	10.00	10.00	10.00
Soybean meal [b]	13.50	13.50	13.50
Gelatin [c]	3.00	3.00	3.00
Feather meal [d]	2.00	2.00	2.00
Blood meal [e]	5.50	5.50	5.50
Pea protein isolate [f]	10.00	10.00	10.00
Wheat gluten [g]	23.00	23.00	23.00
Wheat starch [g]	19.50	19.50	19.50
Linseed oil [h]	0.36	0.36	0.36
Rapeseed oil [i]	9.57	9.57	9.57
Vitamin mineral premix [j]	0.50	0.50	0.50
Lysine [k]	1.20	1.20	1.20
Calcium hydrogen phosphate [l]	0.50	0.50	0.50
TiO_2 [m]	1.00	1.00	1.00
Inert filler [n]	0.37	0.37	0.37
Resveratrol [o]	**-**	**0.30**	**-**
Genistein [o]	**-**	**-**	**0.30**
Nutrient composition [% DM]			
Dry matter	89.41	88.65	88.53
Crude protein	53.60	53.69	53.96
Crude lipid	14.85	14.86	14.72
Crude ash	5.73	5.74	5.68
Total carbohydrates [p]	25.82	25.70	25.64
Gross energy [MJ kg^{-1} DM]	22.80	22.75	22.77

[a] Vereinigte Fischmehlwerke Cuxhaven GmbH & Co. KG, Cuxhaven, Germany; [b] SOYAFINE 200, EURODUNA Rohstoffe GmbH, Barmstedt, Germany; [c] Gustav Ehlert GmbH & Co. KG, Verl, Germany; [d] GoldMehl ©FM, GePro Geflügel-Protein Vertriebsgesellschaft mbH & Co. KG, Diepholz, Germany; [e] Blood meal, Sonac, Son, Netherlands; [f] Emsland-Stärke GmbH, Emlichheim, Germany; [g] KRÖNER STÄRKE GmbH, Ibbenbüren, Germany; [h] Makana Produktion und Vertrieb GmbH, Offenbach a.d.Queich, Germany; [i] Different food stores, Büsum, Germany; [j] Emsland-Aller Aqua GmbH, Golßen, Germany; [k] Biolys, Evonik Industries AG, Essen, Germany; [l] J. Rettenmaier und Söhne GmbH und Co. KG, Rosenberg, Germany; [m] Titanium dioxide: Kronos Titan GmbH, Nordenham, Germany; [n] Bentonite: Del Lago Bentonite, Castiglioni Pes y Cía., Buenos Aires, Argentina; [o] CHEMOS GmbH & Co. KG, Regenstauf, Germany; [p] Total carbohydrates = 1000 - (crude protein + crude fat + crude ash)

2.1.3. Sampling

For the collection of the fecal samples, trout were stripped on a daily basis as described by Austreng (1978). The intestinal passage time of the food given in one feeding event had been determined in a pre-trial. Thus the time point of stripping was adjusted to the timeframe determined and occurred with an offset of 29.5 hours post feeding. The fish were anesthetized in a separate tank using clove oil (concentration: 1 drop/2 L holding water, Bombastus-Werke AG, Freital, Germany), sorted by VIE tag color code and stripped. Afterwards, the fish were returned to the holding tank to recover from anesthetic. Fecal samples that were collected within the first 10 days of the experimental period were discarded. The fecal samples from the following experimental days (day 11 to 22) were pooled according to the VIE tag color code and stored at -20 °C until further analysis.

At the end of the experiment, nine fish of each feeding group were sacrificed for the collection of histological samples. Fish were dissected, grossly examined, and the hindgut immediately preserved in 4% phosphate-buffered formalin (Roti®-Histofix 4%, Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

2.2. Measurement of nutrient composition

The nutrient composition was determined in all experimental diets and the fecal samples. First, frozen fecal samples were freeze-dried (Alpha 1-2 LDplus and Alpha 1-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until the weight was stable. All samples were homogenized using a mortar and pestle and stored at 4 °C. Analysis of the nutrient composition was done according to the EU guideline (EC) 152/2009 (European Union, 2009). For the determination of DM, the homogenized samples were dried at 103 °C in a drying oven until the weight remained stable. Ash content was determined after 4 h incineration at 550 °C in a combustion oven (P300, Nabertherm, Lilienthal, Germany). The Kjeldahl method (InKjel 1225M, WD30, Behr, Düsseldorf, Germany) was used for the determination of the crude protein content (nitrogen x 6.25). The crude lipid content was quantified following extraction with petroleum ether in a Soxleth extraction system (Soxtherm, Hydrotherm, Gerhardt, Königswinter, Germany). The diet samples were hydrolyzed with hydrochloric acid prior to the lipid extraction. Gross energy was measured using a bomb calorimeter (C 200, IKA, Staufen, Germany). Total carbohydrates were calculated according to the formula in Table III- 1.

2.3. Measurement of TiO_2 content and calculation of ADC

The amount of inert marker TiO_2 was measured in the diet as well as the fecal samples according to DIN EN ISO 11885 (2009).

The apparent digestibility coefficient (ADC) of dietary nutrients and gross energy (Table III- 2) were calculated based on the formula by Maynard and Loosli (1969):

$$ADC\ (\%) = 100 \times \left[1 - \frac{(dietary\ TiO_2 \times fecal\ nutrient\ content)}{(fecal\ TiO_2 \times dietary\ nutrient\ content)}\right]$$

2.4. Histopathological analysis

The hindgut samples were taken out of the buffered formalin, embedded in paraffin, cross-sectioned at 3 µm, and stained with hematoxylin and eosin (HE). The analysis of the cross-sections was carried out with a light microscope (Primo Star iLED, Carl Zeiss Microscopy GmbH, Göttingen, Germany) equipped with an 10x eyepiece and 4x/0.10, 10x/0.25, and 40x/0.65 objectives. The tissue morphology of all cross-sections was categorized following an observer-blinded assessment based on Baeverfjord and Krogdahl (1996) and Romarheim et al. (2008) (Table III-3).

2.5. Statistical analysis

All statistical analysis was performed using R (version 3.1.3) with an RStudio interface. The packages *gdata, multcomp, gplots, SimComp,* and *piecewiseSEM* were used for the graphical and statistical analysis.

The data evaluation of the ADC values started with the definition of an appropriate statistical model for normally distributed and homoscedastic data (based on graphical residual analysis). The linear model included the supplement type (none, resveratrol, genistein) as influencing factor. Based on this model, analyses of multiple contrast tests according to Hasler and Hothorn (2008) were performed in order to compare the effects of the three feeding groups.

3. Results

3.1. Resveratrol and genistein slightly affected the digestibility of macronutrients

No mortalities occurred throughout the experimental period and the mean final body weight of rainbow trout did not differ between the dietary treatments (181.2 ± 18.7 g). The apparent digestibility of the experimental diets was slightly but significantly affected by the dietary supplementation of resveratrol and genistein (Table III- 2). Dietary genistein (diet +G) decreased the ADCs of dry matter, crude lipid and gross energy. The ADC of dry matter was significantly reduced from 79.8 ± 1.2% (CD) to 77.3 ± 0.8% (+G; $p > 0.05$, Table III- 2). The ADC of crude lipid was significantly reduced from 93.3 ± 0.6% (CD) to 90.9 ± 0.4% (+G) and the ADC of gross energy was reduced from 86.5 ± 0.8% (CD) to 84.3 ± 0.4% (+G), respectively (both with $p < 0.05$, Table III- 2). Dietary resveratrol (diet +RV) significantly reduced the digestibility of gross energy from 86.5 ± 0.8% in the fish fed the control diet (CD) to 85.0 ± 0.4% when the fish were fed the resveratrol supplemented diet (+RV; $p < 0.05$, Table III- 2).

Table III- 2. Apparent digestibility coefficient (ADC) of macronutrients of rainbow trout fed three experimental diets for three weeks: a control diet (CD), a diet supplemented with resveratrol (+RV; 0.3% DM of diet), and a diet supplemented with genistein (+G; 0.3% DM of diet).

ADC [%]	CD	+RV	+G
Dry matter	79.8 ± 1.2 ^{a}	78.6 ± 0.6 ^{ab}	77.3 ± 0.8 ^{b}
Crude protein	90.6 ± 0.5 $^{+}$	89.8 ± 0.2	89.6 ± 0.4 $^{+}$
Crude lipid	93.3 ± 0.6 ^{a}	92.1 ± 1.0 ^{ab}	90.9 ± 0.4 ^{b}
Gross energy	86.5 ± 0.8 ^{a}	85.0 ± 0.4 ^{b}	84.3 ± 0.4 ^{b}

Values (mean ± SD, n=3) within one row with different superscript letters are significantly different with a *p*-value < 0.05 based on ANOVA as described in Materials and methods. Tendency towards a statistical difference between groups based on a *p*-value < 0.1 is marked by +.

3.2. Dietary phytochemicals did not alter the microscopic histopathology in the intestine

The histopathological analysis of the hindgut revealed no apparent differences in the micromorphology between the feeding groups (CD, +RV, and +G), but rather individual differences (Table III-3). The general intestinal epithelial morphology is depicted in the representative images (Figure III-1). The muscularis externa (ME) was intact and lined by the regular and mostly thin intestinal submucosa (SM) (Table III- 3). The stratum granulosum (SG) consisted of regularly packed cells and the stratum compactum (SC) was slightly folded in all samples. Internal foldings (IF) consisting of circular muscle (annulo-spiral septum (Burnstock, 1959)), could be observed within the intestinal lumen of

all cross-sections (Figure III- 1 A). The structure of the mucosal folds (MF) was very complex with long and partly divided folds (medium to tall height, Table III- 3). The width of the lamina propria (LP) was thin to moderate and showed only slight infiltration of cells in single individuals (Table III- 3). The enterocytes of the mucosal epithelium (EP) had a moderate to high level of vacuolization (Figure III- 1 B, D; Table III- 3). Apart from only one exception, the nuclei of the enterocytes were at a basal position in the tissue section samples of all individuals.

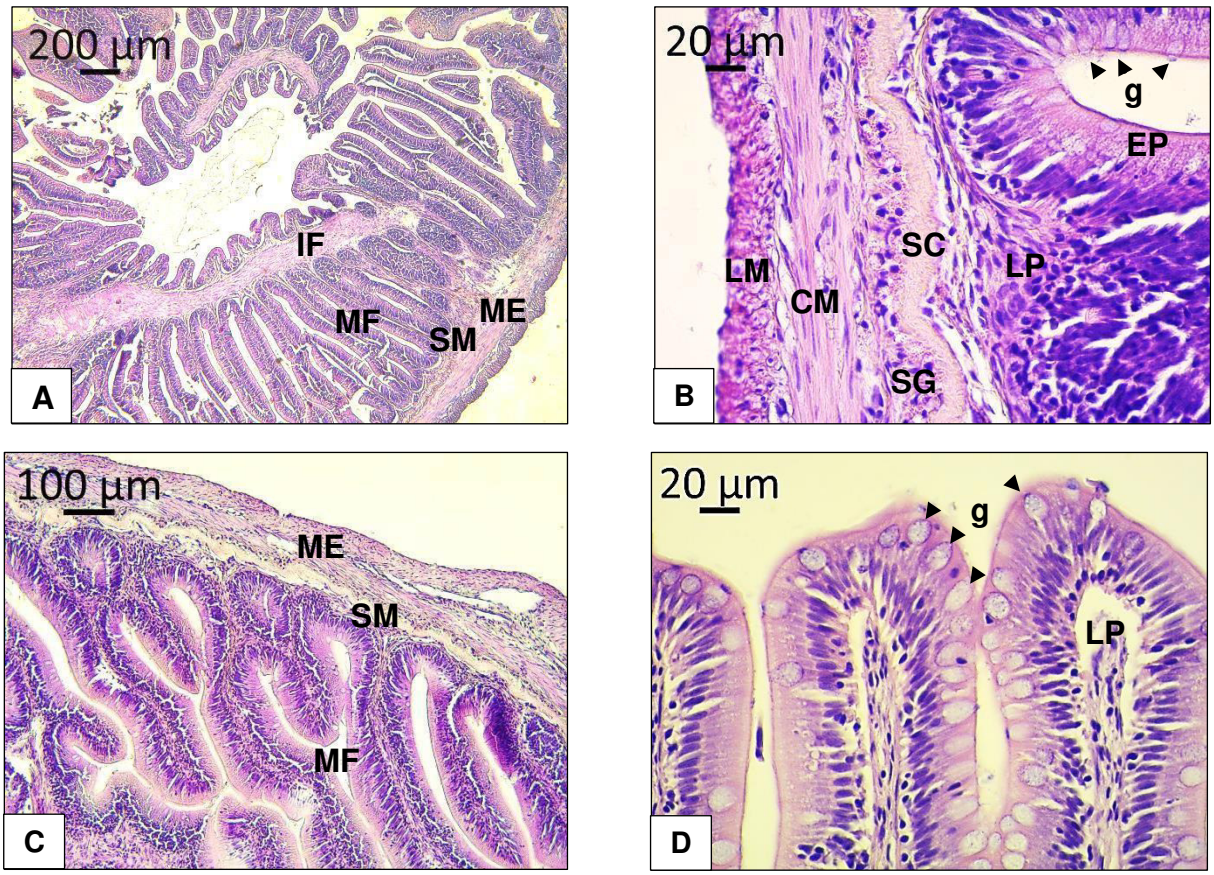

Figure III- 1. Representative images of the intestinal epithelium of rainbow trout fed the experimental diets (the control diet CD, diet +RV that was supplemented with 0.3% DM resveratrol, and diet +G that was supplemented with 0.3% DM genistein) for three weeks. White light images of the intestinal epithelia of fish fed **(A)** diet +G (HE, x 40); **(B)** fed diet CD (HE, x 400); **(C)** fed diet +RV (HE, x 100); **(D)** fed the diet CD (HE, x 400). Abbreviations: muscularis externa (ME), longitudinal muscle (LM), circular muscle (CM), stratum granulosum (SG), stratum compactum (SC), submucosa (SM), lamina propria (LP), mucosal fold (MF), mucosal epithelium (EP), internal folding (annulo-spiral septum) consisting of circular muscle (IF), goblet cells (g).

Table III- 3. Histopathological alterations in the hindgut of rainbow trout fed the experimental diets CD (control diet), +RV (diet supplemented with 0.3% DM resveratrol), and +G (diet supplemented with 0.3% DM genistein) for three weeks. The micromorphology is categorized by the degree of change of each parameter for each single individual (n = 9 for each feeding group).

Group	Mucosal folds, height			Submucosa		Lamina propria, width			
	Short	Med	Tall	Thin	Wide	Thin	Inter	Mod	Wide
CD	0	2	7	7	2	5	3	0	0 *
+RV	0	4	3 *	7	2	5	1	1	0 *
+G	0	1	8	8	1	6	2	1	0

	Enterocyte vacuolization			Nuclear position in enterocyte		Lamina propria, infiltration			
	Low	Mod	High	Basal	Apical displacement	None	Slight	Mod	Marked
CD	1	5	3	9	0	7	2	0	0
+RV	0	4	5	9	0	6	2	0	0 *
+G	1	2	6	8	1	6	3	0	0

Med = medium, Mod = moderate, Inter = intermediate, * = missing evaluation due to poor sample quality.

4. Discussion

The trial revealed slightly but significantly reduced ADCs of dry matter, crude lipid, and gross energy in rainbow trout that were fed the diets supplemented with genistein (diet G) and a significantly reduced ADC of gross energy when resveratrol was supplemented (diet RV, both Table III- 2). To prevent effects on the digestibility deriving from different feeding rations (Bureau et al., 2002; Storebakken and Austreng, 1987), all experimental groups were fed equal daily rations (1.4% of body weight). Thus the reduced digestibility was solely caused by the phytochemicals and not through differences in the feed intake. The slight but significant adverse effect of dietary genistein on several ADCs might indicate an overall impairment of nutrient digestibility. Atlantic salmon (*Salmo salar*) that are fed soybean meal containing high levels of phytochemicals and ANFs exhibit an overall decreased feed conversion ratio and impaired protein utilization (Rumsey et al., 1994). When extracts from soybean, that contain alcohol soluble ANFs, are fed to Atlantic salmon, the digestibility of fat is significantly reduced (Olli and Krogdahl, 1995). In addition, rainbow trout that are fed diets with soybean meal display reduced protein and energy retention and reduced growth rates (Kaushik et al., 1995). These studies support the findings of the reduction of the ADCs of dry matter, gross energy, and crude lipid due to dietary genistein as observed in the current digestibility experiment. In general, the reduction in ADCs caused by resveratrol and genistein ingestion was rather minor (between 1.5% and 2.5%; Table III- 2). Similarly reduced ADCs of dry matter, crude protein, and crude lipid by < 5% are observed when European sea bass are fed tannin-supplemented diets (Omnes et al., 2017). Dietary resveratrol only significantly

affected the digestibility of gross energy. Further studies indicate that resveratrol specifically targets the lipid accumulation and metabolism in various species (Arichi et al., 1982; Caro et al., 2017; Chang et al., 2016).

Ricketts et al. (2005) and Rupasinghe et al. (2016) review the mechanisms of action of different phytochemicals, including resveratrol and genistein, on the impairment of nutrient digestibility and tissue lipid deposition. It is likely that genistein interacts with the energy metabolism *via* AMP-activated protein kinase (AMPK) (Rupasinghe et al., 2016), or reduces the deposition of adipose tissue, which has been observed in mice (Kim et al., 2006; Naaz et al., 2003). Possibly, genistein modulates the expression of genes that encode proteins controlling nutrient retention, as described by Cleveland and Manor (2015). Resveratrol decreases the deposition of lipids into tissues and cells in mice (Chang et al., 2016), murine hepatocytes (Momchilova et al., 2014), and rats (Arichi et al., 1982). Furthermore, dietary administration of wine lees that contain significant amounts of resveratrol among other bioactives lead to a reduction of the lipid content in zebra fish (*Danio rerio*) embryos (Caro et al., 2017). Although the determination of the specific underlying mechanisms were beyond the scope of this study, the reduced gross energy digestibility caused by dietary resveratrol and genistein might, based on the above-mentioned mechanisms, lead to reduced growth (Kaushik et al., 1995; Mambrini et al., 1999; Omnes et al., 2017). Digested energy might be further lost *via* urine, gill excretion, heat increment, and metabolic processes, thus reducing the retained energy available for growth (Bureau et al., 2002; Cho and Bureau, 1995; Jobling, 1994). The short experimental period of this trial prohibits the proper investigation of effects of the phytochemicals on the growth of the rainbow trout. Nevertheless, the decreased ADCs as observed in this study might add to the impaired growth predominantly caused by reduced feed intake in our previous trial (Torno et al., 2017). Own investigations revealed that both phytochemicals can also impair the nutrient utilization and alter the body composition of rainbow trout (Torno et al. unpublished results, see **chapter II**). A limitation of this study might be the unfeasibility to assess the energy and protein retention. This might be realized in future studies by the determination of whole body nutrient composition and the determination of retained energy using respirometer systems (Cho and Bureau, 1995; Steinberg et al., 2017; Stiller et al., 2013).

The microscopic evaluation of the hindgut of rainbow trout revealed no signs of severe histopathological lesions or intestinal inflammation throughout all feeding groups. The general morphology (complex mucosal folds and internal folding) was typical for trout (Burnstock, 1959). An increase in the height and complexity of the mucosal folds and a higher abundance of mucus producing goblet cells is assumed to improve the digestibility of plant ingredients in many fish species (Borgeson, 2006; Escaffre et al., 2007; Estensoro

et al., 2016; Omnes et al., 2015; Tusche et al., 2011; van den Ingh et al., 1991). Mild histopathological alterations, like a slight increase in the width of the lamina propria and the presence of infiltrating cells, were observed in single individuals but were not clearly assignable to one feeding group (Table III- 3). A slightly increased enterocyte vacuolization, that may be induced by dietary soybean compounds (Burrells et al., 1999), was observed in single individuals fed the diet +G (Table III- 3). Severe signs of enteritis induced by soybean or soybean-ANFs (Bureau et al., 1998; Escaffre et al., 2007; Romarheim et al., 2008; van den Ingh et al., 1996) were absent in the rainbow trout from this trial. The 13.5% DM of soybean meal that was utilized in the diet formulation in our current study was markedly less than the amounts associated with enteritis (> 30% in Romarheim et al. (2008)). Even the addition of 0.3% DM genistein did not induce clear signs of enteritis. Dietary resveratrol caused neither damage, nor improvement of the intestinal morphology in this study (Table III- 3 and Figure III- 1). In tilapia (*Oreochromis niloticus*) dietary resveratrol leads to deformation of the intestinal tissue and to irregular cell structure (Zheng et al., 2017). In mice and rats, resveratrol reduces signs of intestinal inflammation and seems to protect the intestine from damage (Larrosa et al., 2009; Sánchez-Fidalgo et al., 2010; Zhang et al., 2017). Furthermore, resveratrol improves the intestinal integrity of European sea bass by reducing the level of inflammatory cytokines (Magrone et al., 2016). Distinct beneficial effects of a diet containing 0.3% DM resveratrol were not observed within this study.

The present data suggest that other reasons besides intestinal inflammation of the hindgut may have caused the reduced digestibility of lipid, dry matter, and gross energy. It cannot be fully ruled out, that resveratrol and genistein might affect other sections of the intestine, though signs of diet-induced enteritis are normally present in the distal intestine (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2000; van den Ingh et al., 1991). Furthermore, bioactives might affect digestive enzymes (McDougall et al., 2008; Ozdal et al., 2013; Stojadinovic et al., 2013), other digestive organs (Sargent et al., 2002), and molecular signaling pathways that are involved in nutrient utilization and metabolism (Kim et al., 2004; Naaz et al., 2003; Zang et al., 2006). A limitation of this study might be the duration of the experiment, which lasted 14 days in terms of acclimatization followed by 22 experimental days. With this experimental set-up we focused on the investigation of potential quickly visible adverse effects of resveratrol and genistein on ADCs and gastrointestinal morphology of rainbow trout. We assume that the duration was efficient to investigate possible morphological changes of the hindgut, since the first signs of dietary induced gastro-intestinal inflammation are generally visible within two to five days in salmonids (Baeverfjord and Krogdahl, 1996; Romarheim et al., 2008).

5. Conclusion

The use of the plant-derived bioactives resveratrol and genistein revealed an impairment of nutrient digestibility but did not affect intestinal morphology of rainbow trout. ADC of gross energy was significantly reduced by dietary resveratrol and genistein, whereas ADCs of crude lipid and dry matter were solely diminished due to genistein. Both phytochemicals exhibited neither beneficial nor detrimental effects on the intestinal morphology. Although the slightly reduced digestibility of macronutrients due to resveratrol and genistein may be considered as an adverse side effect, lesions of the hindgut most likely do not contribute to the antinutrient effects of both phytochemicals. The combined investigation of reduced digestibility and reduced feed intake caused by the phytochemicals and nutrient utilization remains to be elucidated in following studies.

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CHAPTER IV

Effects of low-fish oil diets, temperature, and dietary resveratrol on growth, fatty acid composition, and selected gene-expression of gilthead sea bream (*Sparus aurata*)

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Abstract

In order to sustainably produce fish with a high lipid quality rich in omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFAs), alternative sources of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) need to be identified. The use of bioactive compounds that stimulate the *in vivo* fatty acid synthesis might be a promising tool, thus resveratrol (RV) was investigated as potential fish oil complement in this study. Juvenile gilthead sea bream (*Sparus aurata*) were fed four experimental diets containing either of two fish oil levels (diet F6: 6% dry matter (DM) fish oil; diet F2: 2% DM fish oil) and supplemented with 0.15% DM resveratrol (F6, F2, F6 + RV, F2 + RV) for two months. To determine effects of water temperature on the response of investigated parameters, all four diets were fed to fish challenged either at 19 °C or 23 °C, resulting in a total of eight experimental treatments. Fish held at 23 °C had significantly increased feed intake and growth rates, resulting in increased crude lipid and gross energy content irrespective of the dietary fish oil level or RV supplementation. The fatty acid compositions of whole body homogenate, fillet, and liver tissues were significantly affected by the holding temperature and dietary fish oil level. The feeding of the F6 + RV diet resulted in elevated EPA and DHA contents whereas feeding of the diet F2 + RV resulted in decreased EPA and DHA levels in the livers of sea bream held at 19 °C in comparison to the control diets F6 and F2, respectively. This bi-directional effect may be partly explained by alterations of the mRNA steady state levels of the Δ6-desaturase gene (increased by feeding F2 and F6 + RV diets in comparison to F6) and β-oxidation related genes (increased by feeding of F2 diet compared to F6). This study adds to the basic understanding of nutritional and biochemical regulation of the fatty acid synthesis in the marine sea bream.

Key words: stilbene, EPA, DHA, LC-PUFA, bioactive, fish oil complement, omega-3

1. Introduction

Fish are the predominant source of the omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFAs) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) which play a unique role in human nutrition, health, and development (Tocher, 2015). To produce fish rich in EPA and DHA, aquafeeds contain fish meal and fish oil obtained from wild catches. Current trends demanding a more sustainable and economic fish production, have led to increased development and inclusion of alternative terrestrial and plant ingredients in aquafeeds. Depending on the alternative ingredients used, health and nutritious properties of the farmed fish may be affected (FAO, 2014). The replacement of dietary fish oil by vegetable oils might be feasible in some species, but can be problematic especially in carnivorous marine species (FAO, 2014). The gilthead sea bream (*Sparus aurata*) is a carnivorous marine fish species of economic importance especially in the Mediterranean region (Oliva-Teles, 2000). Sustainable diet formulations with low levels of fish oil do not necessarily affect the growth and performance, but impair the fillet quality and fatty acid (FA) composition of sea bream (Benedito-Palos et al., 2009; Izquierdo et al., 2005; Vasconi et al., 2017). The FA composition of marine species usually reflects that of their diet, since the ability to convert dietary C18 precursor fatty acids to LC-PUFAs is hardly present in marine finfish (Izquierdo et al., 2003; Menoyo et al., 2004). Nowadays, farmed gilthead sea bream can have decreased EPA and DHA contents in comparison to past years and in comparison to wild specimen due to this practice of fish oil replacement (Vasconi et al., 2017).

Considerable effort has been made to prevent this trend. Amongst others, the use of finishing diets high in fish oil to recover the EPA and DHA levels and to wash out short-chained FA is common and has been successfully applied in sea bream (Benedito-Palos et al., 2009; Izquierdo et al., 2005). Furthermore, the use of microalgae containing LC-PUFA in the diets of marine finfish are a promising approach shown in the case of *Pavlova viridis* and European sea bass (*Dicentrarchus labrax*) (Haas et al., 2015). The use of *de novo* n-3 oils from genetically modified oil crops for example in Atlantic salmon (*Salmo salar*) and sea bream (Betancor et al., 2017, 2016) is innovative, but due to legislations not practicable in all countries.

The aforementioned approaches have in common, that they rely on already existing sources of EPA and DHA which are supplied to the fish *via* the diet. Interesting would be an improvement of the innate ability of the fish to cope with diets low in EPA and DHA. The modification of underlying molecular mechanism and exploitation of genetic capacities build the backbone for this approach. In freshwater fish that have a limited natural ability to convert the precursor C18 FA α-linolenic acid (ALA, 18:3n-3) to EPA and

DHA, bioactive phytochemicals might stimulate this FA synthesis. An elevation of EPA and DHA levels has successfully been shown in rainbow trout (*Oncorhynchus mykiss*) fed diets containing resveratrol (Torno et al., 2017) and sesamin (Trattner et al., 2008a), in zebra fish (*Danio rerio*) embryos exposed to wine polyphenols (Caro et al., 2017), and salmon hepatocytes treated with genistein (Schiller Vestergren et al., 2011) and sesamin (Trattner et al., 2008b). Apart from the hardly present *in vivo* FA bioconversion in marine fish, some species like sea bream and sea bass seem to possess the genetic capacity to perform the synthesis at least partly (González-Rovira et al., 2009; Izquierdo et al., 2008; Robin and Skalli, 2007; Tocher and Ghioni, 1999; Turkmen et al., 2017). Thus, it might be possible to exploit the genetic capacity of marine finfish and activate the expression of dormant genes encoding proteins involved in the FA synthesis. Bioactive secondary plant compounds that increased the endogenous FA synthesis of freshwater fish might be a promising tool to be investigated in marine finfish.

Resveratrol (RV) is a stilbene derivate produced by plants, mainly grape vines, in response to infections (Gresele et al., 2011) and has potential health-beneficial, anti-inflammatory, and anti-oxidant properties (Brisdelli et al., 2009; Rupasinghe et al., 2016; Soleas et al., 2001). Interesting for the application in fish are the possible modulation of animal lipid metabolism (Arichi et al., 1982; Caro et al., 2017; Kimura et al., 1983) and the potential to increases elongase and desaturase (∆6- and ∆5-desaturase) activities (Momchilova et al., 2014). The fact that RV indicated LC-PUFA increasing properties in mammalian cell cultures (Frémont et al., 1999; Momchilova et al., 2014), zebra fish (Caro et al., 2017), and rainbow trout (Torno et al., 2017) makes it an interesting target phytochemical for fish nutrition studies with marine finfish.

Additionally, environmental factors may influence the content of n-3 LC-PUFA or the expression and activity of enzymes involved in the FA synthesis in fish (Skalli et al., 2006; Tocher et al., 2004; Vagner and Santigosa, 2011). In freshwater fish and salmonids it seems that the FA desaturation, elongation, and β-oxidation activities are increased at lower temperatures (Tocher et al., 2004; Vagner and Santigosa, 2011). Studies with marine fish reveal controversial results. Vagner et al. (2007) demonstrated that temperature did not affect the diet-induced upregulation of the ∆6-desaturase (∆6-D) in sea bream larvae. In contrast to that, Skalli et al. (2006) demonstrated that the amount of LC-PUFAs was increased in the phospholipid fraction in sea bass held at low water temperatures of 22 °C. Since temperature affects diet intake and growth, it seems likely that the FA synthesis might eventually be affected. Thus, an investigation of phytochemical-induced effects in sea bream under the influence of nutritional and environmental factors is highly interesting.

The aim of this study was to investigate the effects of low-fish oil diets supplemented with dietary RV on (1) growth, (2) performance parameters, (3) whole body nutrient composition, (4) FA composition of the whole body, liver and fillet, and (5) the mRNA steady state levels of selected genes encoding proteins involved in the desaturation (Δ6-D), FA metabolism (Carboxyl ester lipase (CEL)), and β-oxidation of FAs (Peroxisome proliferator-activated receptor α (PPARα); Enoyl-CoA hydratase (ECH)) of the gilthead sea bream. Furthermore, we addressed the question whether the rearing temperature played a role in the response of the investigated parameters.

2. Materials and Methods

2.1. Experimental diets

Four different experimental diets (isonitrogenous and isoenergetic) were formulated as shown in Table IV- 1. All diets consisted of mainly alternative plant protein sources (Soybean concentrate, corn gluten, wheat gluten, and rapeseed expeller) and had a fish meal content of 5% dry matter (DM). Diet F6 contained 6% DM fish oil and 3% DM of a mixture of vegetable oils (linseed, rapeseed, and palm oil). F6 served as overall reference diet. Diet F2 had a fish oil content of 2% DM and contained 7% DM of a mixture of linseed, rapeseed, and palm oil. The diets contained considerably different amounts of the fatty acids EPA and DHA as shown in Table IV- 2. Basal diet F6 was formulated to meet the recommended amount of EPA and DHA for sea bream (EPA + DHA: 0.9% of dry diet when DHA/EPA = 1 (Kalogeropoulos et al., 1992; NRC, 2011)). Basal diet F2 contained reduced amounts of EPA and DHA (0.37% of dry diet, Table IV- 2) below the recommendation for sea bream. Diets F6 + RV and F2 + RV were equal to diets F6 and F2, respectively, and supplemented with resveratrol (+RV; trans-3,4',5-trihydroxy stilbene, purity ≥ 98%, Chemos GmbH & Co. KG, Regenstauf, Germany) at a concentration of 0.15% DM of diet. The dietary essential amino acid (EAA) content of each diet was calculated from the EAA content of the single ingredients. All diets were formulated based on the macronutrient and the EAA requirements of gilthead sea bream according to Peres and Oliva-Teles (2009) and Wilson (2003). All diets were pressed into 3- and 4-mm pellets using a feed press L14-175 (Amandus Kahl, Reinbek, Germany).

Table IV- 1. Ingredients and nutrient composition (percentage of dry matter: % DM) of the experimental diets. F6 and F2 are the basal diets with 6% and 2% DM fish oil, respectively. +RV indicates the supplementation of the basal diets with 0.15% DM resveratrol.

Ingredients [% DM]	F6	F2	F6 + RV	F2 + RV
Fish meal (*Clupea sp.*) [1]	5.00	5.00	5.00	5.00
Soybean concentrate (HP 300) [2]	19.00	19.00	19.00	19.00
Blood meal [3]	7.00	7.00	7.00	7.00
Corn gluten [2]	18.00	18.00	18.00	18.00
Wheat gluten [4]	12.50	12.50	12.50	12.50
Rapeseed expeller [5]	8.00	8.00	8.00	8.00
Wheat starch [4]	10.96	10.96	10.96	10.96
Fish oil [1]	6.00	2.00	6.00	2.00
Linseed oil [6]	2.00	2.08	2.00	2.08
Rapeseed oil [7]	0.10	1.65	0.10	1.65
Palm oil [8]	0.90	3.27	0.90	3.27
Vitamin Mineral premix [9]	0.50	0.50	0.50	0.50
Methionine [9]	0.85	0.85	0.85	0.85
Lysine [10]	1.19	1.19	1.19	1.19
Di-calcium phosphate [11]	2.08	2.08	2.08	2.08
Inositol [12]	0.02	0.02	0.02	0.02
Choline chloride [13]	0.13	0.13	0.13	0.13
Cholesterol [12]	0.11	0.11	0.11	0.11
Lecithin [12]	2.53	2.53	2.53	2.53
α-Cellulose [14]	2.00	2.00	2.00	2.00
Inert filler [15]	1.13	1.13	1.13	1.13
Resveratrol (RV) [16]	-	-	0.15	0.15
Nutrient composition [% DM]				
Dry matter	90.7	90.9	90.8	91.1
Crude ash	7.1	6.9	7.1	6.9
Crude protein	49.3	49.7	49.7	49.5
Crude lipid	14.5	14.6	14.3	14.8
Total carbohydrates	29.1	28.8	28.9	28.8
Gross energy [MJ kg^{-1} DM]	22.89	22.83	22.90	22.91

[1] Vereinigte Fischmehlwerke Cuxhaven GmbH & Co. KG, Cuxhaven, Germany; [2] EURODUNA Rohstoffe GmbH, Barmstedt, Germany; [3] Sonac, Son, Netherlands; [4] KRÖNER STÄRKE GmbH, Ibbenbüren, Germany; [5] Stöfen Landhandel, Wesselburen, Germany; [6] Makana Produktion und Vertrieb GmbH, Offenbach a.d.Queich, Germany; [7] Different food stores, Büsum, Germany; [8] EFG Elbe Fetthandel GmbH, Geesthacht, Germany; [9] Emsland-Aller Aqua GmbH, Golßen, Germany; [10] Biolys: Evonik Industries AG, Essen, Germany; [11] J. Rettenmaier und Söhne GmbH und Co. KG, Rosenberg, Germany; [12] Roth GmbH, Karlsruhe, Germany; [13] Sigma Aldrich, St. Louis, USA; [14] Mikro-Technik GmbH & Co. KG, Bürgstadt/Main, Germany; [15] Bentonite: Del Lago Bentonite, Castiglioni Pes y Cía., Buenos Aires, Argentina; [16] CHEMOS GmbH & Co. KG, Regenstauf, Germany

Table IV- 2. Fatty acid composition (in % of total fatty acid methyl esters (FAMEs) and % of dry matter of diet (% DM)) of the experimental diets. F6 and F2 are the basal diets containing 6% and 2% DM fish oil, respectively. +RV indicates the supplementation of the basal diets with 0.15% DM resveratrol. The standard used for identification of individual FAMEs consisted of all 12 FAMEs shown here.

[% of FAMEs]	F6	F2	F6 + RV	F2 + RV
14:0	2.66	1.31	2.58	1.30
16:0	19.29	27.82	19.41	27.98
18:0	3.60	3.74	3.56	3.74
Σ SFA [1]	**25.55**	**32.87**	**25.54**	**33.02**
16:1n-7	2.83	1.16	2.67	1.13
18:1n-7c	2.51	1.90	2.44	1.90
18:1n-9c	24.54	24.72	24.11	24.73
Σ MUFA [2]	**29.88**	**27.78**	**29.22**	**27.76**
18:2n-6c (LA) [3]	24.80	24.19	25.24	24.20
18:3n-3 (ALA) [4]	11.77	12.11	12.48	12.03
18:3n-6	0.15	0.05	0.13	0.05
20:5n-3 (EPA) [5]	3.13	1.17	3.00	1.18
22:5n-3	0.77	0.26	0.70	0.27
22:6n-3 (DHA) [6]	3.89	1.58	3.63	1.50
Σ PUFA [7]	**44.52**	**39.35**	**45.20**	**39.22**
Σ EPA + DHA	**7.02**	**2.75**	**6.63**	**2.68**
DHA / EPA	1.24	1.35	1.21	1.27
ALA / LA	0.47	0.50	0.49	0.50
Σ EPA + DHA % DM [8]	0.95	0.37	0.88	0.37

[1] ∑ SFA is the sum of saturated fatty acids; [2] ∑ MUFA is the sum of monounsaturated fatty acids; [3] LA: Linoleic acid; [4] ALA: α-Linolenic acid; [5] EPA: Eicosapentaenoic acid; [6] DHA: Docosahexaenoic acid; [7] ∑ PUFA is the sum of n-3 and n-6 polyunsaturated fatty acids; [8] The determination of EPA + DHA % DM of diet was done using the internal standard 13:0 methyl ester and amount of lipid measured in the diet (Table IV- 1).

2.2. Experimental setup

The study was conducted at the facilities of the Grupo de Investigación en Acuicultura, Universidad de Las Palmas de Gran Canaria (GIA-ULPGC), Telde, Las Palmas, Canary Islands, Spain. Two experimental setups were prepared for the feeding trial to realize a simultaneous diet- and temperature-challenge. (1) A flow-through system with 12 tanks was used for the high-temperature challenge (approx. 23 °C). (2) Identical temperature-controlled small recirculating aquaculture systems (RAS) equipped with a total of 12 tanks were used for the low-temperature challenge (approx. 19 °C).

(1) The flow-through system had a natural photoperiod (12 h light) during the whole adaptation and experimental period. The cylindrical fiberglass tanks (500 L) were supplied

with filtered seawater (37‰ salinity) at a rate of 600 L h^{-1} and continuously aerated. Temperature (22.6 ± 0.6 °C), oxygen (6.2 ± 0.3 mg L^{-1}), and pH (8.22; pH-Meter Basic 20+, CRISON, Hach Lange Spain, Barcelona, Spain) were monitored daily. Ammonia (NH_4: < 0.15 mg L^{-1}) and nitrite (NO_2: < 0.1 mg L^{-1}, Royal Ammonia Professional Test and Royal Nitrite Professional Test, Royal Nature, Nesher, Israel) concentrations were determined every second day.

(2) The RAS system had 3 000 L water volume each and were equipped with a cooling unit and a water treatment unit consisting of a sand filter (filtration area: 0.33 m^3; granular size: 0.4 – 1.2 mm, AstralPool, Fluidra, Barcelona, Spain), a multibed bio filter (filtration area: 0.32 m^2; Kripsol, Toledo, Spain), and a protein skimmer. Each RAS had three cylindrical fiberglass tanks (500 L) that were supplied with temperature controlled sea water same as in the flow-through system. The water quality parameters did not differ between the RAS and were measured as described above (temperature: 19.0 ± 1.5 °C; oxygen: 7.1 ± 0.3 mg L^{-1} O_2; pH: 8.06; NH_4: < 1 mg L^{-1}; NO_2: < 0.5 mg L^{-1}).

A total of 600 juvenile gilthead sea bream (offspring from brood stock of GIA-ULPGC, initial body weight: 12.5 ± 2.2 g) were acclimated in the flow-through system and randomly and equally distributed among all 24 tanks of both setups, 25 individuals per tank. Over the first seven days of the experimental period, the RAS were continuously cooled down from 23 °C to 19 °C. The four dietary treatments (F6, F6 + RV, F2, and F2 + RV) were randomly distributed among the twelve tanks of each setup, ensuring that each dietary treatment was tested at each temperature in a triplicate approach. During the whole experimental period of 55 days, the fish were fed manually three times per day until apparent satiation in both setups. The administered feed ratios were determined daily for the calculation of the daily feed intake (DFI).

2.3. Sampling

The tissue samples were collected before the onset of the feeding trial (day 0) and at the end of the trial (day 56). For the initial sampling at day 0, seven fish were sacrificed (pooled sample) and stored at -80 °C for the determination of whole body nutrient composition and fatty acid composition. Additionally, six fish were sacrificed for the collection of liver and fillet tissue samples. The whole liver was weighed (± 0.01 g) for the determination of the Hepatosomatic index (HSI, see formula 6.). For mRNA quantification *via* qRT-PCR, one part of the liver tissue was preserved in RNALater (Sigma-Aldrich, Taufkirchen, Germany). For the determination of the liver fatty acid composition, the remaining liver parts of all six individuals were pooled in one sample and immediately frozen at -80 °C. The fillets (left side) from all six fish were pooled and frozen at -80 °C for the determination of the fatty acid composition. For the final sampling at day 56, five fish

per tank were sacrificed (pooled sample) and stored at -80 °C for the determination of whole body nutrient composition and the fatty acid composition. Additionally, five fish per tank were sacrificed for the collection of the liver and fillet tissue samples. The procedure was the same as described for the initial sampling.

For the determination of growth, performance, and nutrient utilization parameters, all fish were individually weighed (± 0.1 g) and measured (± 0.1 cm) at day 0 and day 56 for the calculation of the initial and final body weight (IBW and FBW, respectively). The specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV), and Fulton condition factor (FCF) were calculated according to the following formulas.

1. $\text{SGR}\ [\%\ \text{d}^{-1}] = \frac{[\ln(\text{FBW}) - \ln(\text{IBW})]}{\text{feeding day}} \times 100$

2. $\text{FCR} = \frac{\text{feed intake [g]}}{\text{weight gain [g]}}$

3. $\text{PER} = \frac{\text{weight gain [g]}}{\text{protein intake [g]}}$

4. $\text{PPV}\ [\%] = \frac{(\text{final body protein [g]} \times \text{FBW [g]}) - (\text{initial body protein [g]} \times \text{IBW [g]})}{\text{protein intake [g]}} \times 100$

5. $\text{FCF} = 100 \times (\text{FBW [g]} \times \text{final body length}[\text{cm}]^{-3})$

6. $\text{HSI}\ [\%] = \frac{\text{liver weight [g]}}{\text{FBW [g]}} \times 100$

2.4. Nutrient composition analysis

The nutrient composition was analyzed in all experimental diets and the whole body homogenates of the gilthead sea bream. Frozen whole body samples were freeze-dried (Alpha 1-2 LDplus and Alpha 1-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until the weight was stable and homogenized using a cutting mill (GM 200, Retsch, Haan, Germany). Diets were homogenized using a mortar and pestle. Analysis of nutrients and gross energy was done according to EU guideline (EC) 152/2009 (European Union, 2009). For the determination of DM, the homogenized samples were dried at 103 °C in a drying oven until the weight remained stable. Ash content was determined after 4 h incineration at 550 °C in a combustion oven (P300, Nabertherm, Lilienthal, Germany). The Kjeldahl method (InKjel 1225M, WD30, Behr, Düsseldorf, Germany) was used for the determination of the crude protein content (nitrogen x 6.25). The crude lipid content was quantified following extraction with petroleum ether in a Soxleth extraction system (Soxtherm, Hydrotherm, Gerhardt,

Königswinter, Germany). The diet samples were hydrolyzed with hydrochloric acid prior to the lipid extraction. Gross energy was measured using a bomb calorimeter (C 200, IKA, Staufen, Germany). Total carbohydrates were calculated according to formula 7.:

7. $$\text{Total carbohydrates} = 1000 - (\text{crude protein} + \text{crude lipid} + \text{crude ash})$$

2.5. Lipid extraction and fatty acid composition analysis

The total lipids were extracted from the whole body homogenates, liver samples, fillet samples, and diet samples according to Folch et al. (1957). The measurement of fatty acid methyl esters (FAMEs) was performed using a gas chromatograph with flame ionization detector (GC-FID; Agilent Technologies, Santa Clara, CA, USA). In brief, total lipids were extracted from liver samples according to Folch et al. (1957). Methylation of fatty acids and extraction of methylated FAMEs was conducted with the help of the Folch reagent (chloroform:methanol 2:1). Samples were neutralized using potassium hydroxide (0.1 M) and FAMEs were isolated by the addition of the Folch reagent and subsequent centrifugation for 10 min at 2000× *g*. The organic phase was collected and a second extraction with potassium hydroxide and the Folch reagent was performed, followed by centrifugation (5 min at 2000× *g*) and drying of samples under a N_2 flux. Re-dissolved FAME samples were injected into a 7820A Agilent gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent HP-88 fused silica capillary column (60 m × 250 µm × 0.2 µm, Agilent Technologies) and helium (1.2 mL/min) as the carrier gas. The following temperature protocol was applied: initial temperature 125 °C, ramp 8 °C/min to 145 °C (26 min), ramp 2 °C/min to 220 °C (5 min). Chromatograms were recorded and analyzed using EZChrom Elite software (Agilent Technologies). A 13-FAME standard was used to identify the retention times of the individual FAMEs (Table IV- 6, 7, and 8). The fatty acid composition was calculated as a percentage of single FAME relative to total FAMEs. The internal standard 13:0 methyl ester was used to calculate FAs as % DM of diet.

2.6. mRNA extraction and qRT-PCR

Based on the results of growth, performance, and FA composition measurement of the mRNA steady state levels was performed only in liver of fish held at 19 °C. Total mRNA was extracted from the liver samples using the Innuprep RNA Mini Kit (Analytik Jena, Germany) according to the manufacturer's protocol. Tissue was homogenized in a TissueLyser II (Qiagen, Hilden, Germany) prior to RNA isolation. RNA concentration and purity were determined *via* NanoDrop measurements (NanoDrop2000c; ThermoScientific,

Waltham, MA, USA) at 260, 280 and 230 nm absorbance. qRT-PCR was performed with a SensiFast SYBR No-ROX One-Step Kit (Bioline, London, UK) on a Rotor-Gene 6000 real-time PCR cycler (Corbett/Qiagen). The primers used and the appropriate annealing temperatures are listed in Table IV- 3. The transcript expression was quantified by calculating the input copy number using a standard curve. Subsequently, respective target mRNA steady state levels of Δ6-D, PPARα, ECH and CEL were normalized to the mRNA levels of the housekeeping gene beta-actin (β-actin). Data are shown as relative mRNA steady state levels of respective target genes normalized to their internal control (β-actin) following absolute quantification (Figure IV- 1).

Table IV- 3. Primer sequences for hepatic mRNA measurements *via* qRT-PCR. Forward and reverse primers as well as their specific annealing temperatures used for qRT-PCR measurements of mRNA levels in total RNA samples extracted from gilthead sea bream liver.

Primer	Sequence 5' → 3'	Annealing temperature (°C)
Δ6-D [a]* forward	GCAGGCGGAGAGCGACGGTCTGTTCC	65
Δ6-D [a]* reverse	AGCAGGATGTGACCCAGGTGGAGGCAGAAG	65
β-actin [b]* forward	TCCTGCGGAATCCATGAGA	60
β-actin [b]* reverse	GACGTCGCACTTCATGATGCT	60
PPARα [c]* forward	TCTCTTCAGCCCACCATCCC	61
PPARα [c]* reverse	ATCCCAGCGTGTCGTCTCC	61
ECH [d§] forward	GCCCAAGAAGCCAAGCAATCAG	60
ECH [d§] reverse	CTTTAGCCATAGCAGAGACCAGTTTG	60
CEL [e§] forward	GCTGAGGAGATTGCTCTGAAGGT	62
CEL [e§] reverse	CAGGAAGCCATAGTCTCACCAGTG	62

[a] Δ6-D: Δ6-desaturase; [b] β-actin: Beta-actin; [c] PPARα: Peroxisome proliferator-activated receptor α; [d] ECH: Enoyl-CoA hydratase; [e] CEL: Carboxyl ester lipase; * Betancor et al. (2016); § Benedito-Palos et al. (2014)

2.7. Statistical analysis

All statistical Analysis was performed using R (version 3.1.3) with an RStudio interface. The packages *gdata, multcomp, gplots, nparcomp, nlme, piecewiseSEM, SimComp*, and *car* were used for the graphical and the statistical analysis.The data evaluation started with the definition of an appropriate statistical model based on a graphical residual analysis of the data and Levene's test to test for homoscedasticity of variances: (1) statistical model based on generalized least squares (*gls*) for normally distributed and heteroscedastic data (IBW, FCR, crude protein) and (2) linear model (*lm*) for normally distributed and homoscedastic data (DFI, FBW, FCF, HSI, SGR, PER, PPV, crude ash, crude fat, gross energy, dry matter, and fatty acid composition). Both models included the level of dietary fish oil content (6% and 2%), supplement (None, RV), and

temperature (19 °C, 23 °C), as well as their interaction term as fixed factors. An analysis of variances (ANOVA) was conducted followed by an appropriate post-hoc test: (1) ANOVA based on *gls* was followed by multiple contrast tests for heteroscedastic data according to Hasler and Hothorn (2008); (2) ANOVA based on *lm* was followed by multiple contrast tests according to Schaarschmidt and Vaas (2009). All post-hoc tests compared the feeding groups based on the fish oil level and supplement within one holding temperature and between the two temperatures.

For mRNA steady state levels (Δ6-D, PPARα, ECH, CEL) the data evaluation was initiated with the definition of an appropriate mixed model (*lme*) with fish oil content (6% and 2%), supplement (None, RV) and their interaction term as fixed factors, and fish tank as random factor. A residual analysis revealed the data to be non-normally distributed. Multiple contrast tests for relative effects were conducted in order to compare the influence of varying factors (Schaarschmidt and Vaas, 2009).

3. Results

3.1. Growth and performance were affected by temperature but not by dietary treatment

During the experimental period of eight weeks, all experimental groups exhibited overall good growth and performance (Table IV- 4). The sea bream held at 19 °C doubled their weight (approx. 2.2 fold) and fish held at 23 °C tripled their weight (approx. 3.4 fold). The SGR of fish held at 19 °C was between 1.3% d^{-1} (F2 + RV) and 1.6% d^{-1} (F6). Fish held at 23 °C had higher SGR with values between 2.2% d^{-1} (F2 + RV) and 2.4% d^{-1} (F6 + RV), respectively. The same applied for the DFI which was about 3.2 for fish held at 19 °C and 4.2 for fish held at 23 °C. Fish of each feeding treatment had significantly lower FBW, SGR, and DFI at 19 °C in comparison to 23 °C ($p < 0.05$, Table IV- 4, indicated by *). The FCR remained unaffected by dietary treatment or temperature challenge and ranged from lower values of 1.9 (F2 + RV at 23 °C) to higher values of 2.5 (F2 + RV at 19 °C). The PER and PPV differed only in tendency between the same dietary treatments held at different temperatures (F2 and F2 + RV). The HSI differed within the same dietary treatment between the two holding temperatures and reached higher values in fish held at 19 °C. When fish were fed the RV-supplemented diets and held at 19 °C, a significantly elevated HSI was present in the group F2 + RV in comparison to the group F6 + RV ($p < 0.05$, Table IV- 4, indicated by m, n). The FCF did not differ between the dietary treatments, but between the two holding temperatures with significantly higher values at 23 °C. The evaluation of the statistical significance of effects proved that all growth and performance parameters were affected by the holding temperature in the first place (Table IV- 5).

Table IV- 4. Growth, nutrient utilization, performance, and final whole body nutrient composition (percentage of original matter (% OM) and MJ kg^{-1} OM) of gilthead sea bream fed with the experimental diets for 8 weeks and held at two different temperatures, 19 °C and 23 °C. F6 and F2 indicate the feeding with the basal diets containing 6% and 2% DM fish oil, respectively. +RV indicates feeding the diets supplemented with 0.15% DM resveratrol.

	19 °C				23 °C				Comparison between 19 and 23 °C			
	F6	F2	F6 + RV	F2 + RV	F6	F2	F6 + RV	F2 + RV	F6	F2	F6 + RV	F2 + RV
IBW [1]	12.4 ± 0.3	12.5 ± 0.3	12.5 ± 0.3	12.8 ± 0.4	12.6 ± 0.2	12.2 ± 0.2	12.7 ± 0.2	12.5 ± 0.1				
FBW [2]	29.7 ± 0.9	27.0 ± 1.7	28.4 ± 1.1	26.0 ± 0.6	42.3 ± 1.3	40.4 ± 0.9	44.1 ± 1.5	40.0 ± 5.0	***	***	***	***
SGR [3]	1.6 ± 0.1	1.4 ± 0.1	1.5 ± 0.0	1.3 ± 0.0	2.3 ± 0.1	2.3 ± 0.0	2.4 ± 0.1	2.2 ± 0.2	***	***	***	***
DFI [4]	3.2 ± 0.2	3.0 ± 0.2	3.3 ± 0.2	3.2 ± 0.3	4.1 ± 0.2	4.3 ± 0.1	4.4 ± 0.0	4.3 ± 0.2	***	***	***	***
FCR [5]	2.1 ± 0.2	2.2 ± 0.1	2.2 ± 0.2	2.5 ± 0.2	1.8 ± 0.0	1.9 ± 0.0	1.9 ± 0.1	1.9 ± 0.1				
PER [6]	1.1 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1				(*)
PPV [7]	16.7 ± 1.8	14.3 ± 3.1	14.9 ± 1.4	13.6 ± 1.5	18.5 ± 1.4	17.7 ± 0.6	17.9 ± 0.7	17.1 ± 1.0		(*)		(*)
HSI [8]	2.4 ± 0.3	2.7 ± 0.1	2.2 ± 0.1 ^{n}	2.7 ± 0.2 ^{m}	2.0 ± 0.1	2.1 ± 0.2	1.7 ± 0.1	2.0 ± 0.3	*	*	*	*
FCF [9]	1.6 ± 0.0	1.6 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.7 ± 0.0	1.7 ± 0.0	1.7 ± 0.0	1.7 ± 0.1	*	*	*	*
Nutrient composition [% OM]												
Dry matter	30.9 ± 0.8	29.6 ± 0.6	29.3 ± 0.2	30.2 ± 0.3	33.5 ± 1.8	32.4 ± 0.6	32.8 ± 1.3	32.1 ± 0.7	*	**	**	(*)
Crude ash	3.8 ± 0.0	3.9 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	3.4 ± 0.1 $^{(b),(B)}$	3.6 ± 0.1 $^{(a)}$	3.6 ± 0.1 $^{(A)}$	3.8 ± 0.1	***	**	**	
Crude protein	16.0 ± 0.2	16.2 ± 0.1	15.9 ± 0.2	16.2 ± 0.3	16.0 ± 0.8	16.3 ± 0.1	15.9 ± 0.4	16.2 ± 0.4				
Crude lipid	10.7 ± 0.8 $^{(a),(A)}$	9.2 ± 0.7 $^{(b)}$	9.1 ± 0.2 $^{(B)}$	9.7 ± 0.4	14.1 ± 0.9 ^{a}	12.4 ± 0.9 ^{b}	13.2 ± 0.7	12.1 ± 0.9	***	***	***	**
Gross energy [10]	8.07 ± 0.32 $^{(A)}$	7.44 ± 0.28	7.32 ± 0.15 $^{(B)}$	7.64 ± 0.12	9.31 ± 0.54	8.73 ± 0.31	9.03 ± 0.49	8.60 ± 0.34	**	**	***	*

[1] IBW = Initial body weight [g]; [2] FBW = Final body weight [g]; [3] SGR = Specific growth rate [% d^{-1}]; [4] DFI = Daily feed intake [% d^{-1}]; [5] FCR = Feed conversion ratio; [6] PER = Protein efficiency ratio; [7] PPV = Protein productive value [%]; [8] HSI = Hepatosomatic index [%]; [9] FCF = Fulton condition factor; [10] Gross energy is given in MJ kg^{-1} OM

Initial nutrient composition: dry matter: 29.7%; crude ash: 4.2% OM; crude protein: 17.0% OM; crude lipid: 8.4% OM; gross energy: 7.34 MJ kg^{-1} OM. Values (mean ± SD, n=3; HSI and FCF: n=15) with different superscript letters and different types of letters within one temperature treatment differ with *p*-values < 0.05 based on ANOVA as described in Materials & methods. Superscript letters indicate the output of tests based on comparisons of fish oil level within one supplement group (a, b: F6 vs. F2; m, n: F6 + RV vs. F2 + RV) and effect of supplementation within one fish oil level group (A, B: F6 vs. F6 + RV) separated by temperature. The statistical output of test based on the effect of the different holding temperatures analyzed within one feeding group are indicated in separate columns using * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. All designations in brackets indicate a tendency towards a difference based on $p < 0.1$.

Table IV- 5. Statistical significance of effects (*p*-values) caused by dietary fish oil level (Oil), resveratrol supplementation (RV), rearing temperature (Temp.), and the interaction of either two factors or all three factors. P-values are given for effects on selected growth and performance parameters, body nutrients, and fatty acids of different tissues of gilthead sea bream at the end of the eight week trial.

		Single effects						Interactions							
		Oil		RV		Temp.		Oil : RV		Oil : Temp.		RV : Temp.		Oil : RV : Temp.	
	FBW [1]	0.005	**	0.811		<0.001	***	0.573		0.786		0.298		0.443	
	SGR [2]	0.003	**	0.280		<0.001	***	0.427		0.254		0.236		0.477	
	DFI [3]	0.461		0.182		<0.001	***	0.470		0.220		0.939		0.082	(*)
	HSI [4]	<0.001	***	0.047	*	<0.001	***	0.141		0.280		0.612		0.630	
	FCF [5]	0.371		0.078	(*)	<0.001	***	0.981		0.514		0.398		0.468	
	Dry matter	0.161		0.200		<0.001	***	0.094	(*)	0.378		0.961		0.245	
	Crude ash	0.014	*	0.006	**	<0.001	***	0.201		0.127		0.233		0.522	
	Crude lipid	0.007	**	0.076	(*)	<0.001	***	0.045	*	0.123		0.916		0.195	
	Gross energy	0.031	*	0.111		<0.001	***	0.069	(*)	0.233		0.817		0.174	
Whole body	Σ SFA [6]	<0.001	***	0.036	*	<0.001	***	0.844		0.489		0.337		0.479	
	Σ MUFA [7]	<0.001	***	0.054	(*)	0.001	***	0.018	*	0.002	**	0.961		0.147	
	Σ PUFA [8]	0.003	**	0.027	*	<0.001	***	0.130		0.058	(*)	0.666		0.536	
	EPA+DHA [9]	<0.001	***	0.136		<0.001	***	0.742		<0.001	***	0.368		0.756	
Liver tissue	Σ SFA	0.029	*	0.583		<0.001	***	0.668		0.890		0.201		0.650	
	Σ MUFA	0.001	**	0.377		0.080	(*)	0.008	**	0.093	(*)	0.762		0.451	
	Σ PUFA	0.105		0.317		<0.001	***	0.026	*	0.193		0.266		0.398	
	EPA+DHA	<0.001	***	0.534		<0.001	***	0.003	**	0.583		0.811		0.018	*
Fillet tissue	Σ SFA	<0.001	***	0.179		<0.001	***	0.364		0.053	(*)	0.587		0.428	
	Σ MUFA	<0.001	***	0.130		0.053	(*)	0.988		0.267		0.237		0.639	
	Σ PUFA	0.007	**	0.061	(*)	<0.001	***	0.679		0.073	(*)	0.205		0.450	
	EPA+DHA	<0.001	***	0.221		<0.001	***	0.720		0.019	*	0.219		0.270	

[1] FBW = Final body weight, [2] SGR = Specific growth rate, [3] DFI = Daily feed intake, [4] HSI = Hepatosomatic index, [5] FCF = Fulton condition factor, [6] SFA = Saturated fatty acids, [7] MUFA = Monounsaturated fatty acids, [8] PUFA = Polyunsaturated fatty acids, [9] EPA+DHA = Sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Significant effects are indicated with * ($p < 0.5$), ** ($p < 0.01$), and *** ($p < 0.001$). A statistical tendency ($p < 0.1$) is indicated by (*).

3.2. Whole body nutrient composition was predominantly affected by temperature and secondly by dietary treatment

After the eight week experimental period, the whole body nutrient composition of fish differed based on the two holding temperatures, except for crude protein (Table IV- 4 and 5). The fish had significantly higher dry matter, crude lipid, and gross energy and significantly lower crude ash when held at 23 °C ($p < 0.05$, Table IV- 4, indicated by *). Additionally, fish held at 23 °C and fed the diet F6 had significantly elevated crude lipid in comparison to fish fed diet F2 ($p < 0.05$, Table IV- 4, indicated by a, b). A similar trend was observed at the lower holding temperature. The RV-supplementation tended to decrease crude lipid and gross energy in fish fed the diet F6 + RV in comparison to fish fed diet F6, both held at 19 °C ($p < 0.1$, Table IV- 4, indicated by (A), (B)). The evaluation of the statistical significance of effects indicated, that crude lipid was predominantly affected by the interaction of dietary fish oil and RV-supplementation (Table IV- 5). Similar tendencies ($p < 0.1$) were visible for gross energy and dry matter.

3.3. Fatty acid composition of different tissues of sea bream was predominantly affected by the holding temperature and the dietary fish oil level

The FA composition of the sea bream whole body homogenates did not fully reflect the FA composition of the diets. Fish fed the diets F6 and F6 + RV had similar amounts of saturated FA (SFA) in comparison to the diet. The amounts of monounsaturated FA (MUFA) were visibly elevated, whereas the amounts of PUFA were visibly diminished in their whole body, liver, and fillet tissues in comparison to the dietary levels (Tables IV- 2, 6, 7, and 8). Apart from the liver tissue, the amount of EPA + DHA was almost the same as in the diets. The FA composition of all tissues analyzed of fish fed the diets F2 and F2 + RV were visibly different from the FA composition of the diet. Generally, the diets consisted predominantly of PUFAs, followed by SFAs and then MUFAs (Table IV- 2). On the contrary, the fish tissue contained MUFAs in the first place, followed by PUFAs and then SFAs, except for liver tissue of fish held at 23 °C (Tables IV- 6, 7, and 8). The relative amount of EPA + DHA only represented that of the diets in the whole body and fillet tissue of sea bream held at 19 °C. In all other cases, the amount of EPA + DHA was visibly reduced in the fish compared to the diet.

3.3.1. Whole body homogenate

The overall FA composition of the whole body homogenate of sea bream challenged with different dietary treatments and different holding temperatures for eight weeks was predominantly affected by the interaction of the holding temperature and the dietary fish oil level (Table IV- 5). Based on different holding temperatures, the amounts of SFAs and

MUFAs were significantly higher in fish held at 23 °C, whereas the amount of PUFAs was significantly lower in those specimens ($p < 0.05$, Table IV- 6, indicated by *). Additionally, the amount of EPA + DHA and also the n-3/n-6 ratio was significantly reduced in fish fed the diets F2 and F2 + RV and held at 23 °C in comparison to the same feeding groups held at 19 °C. Furthermore, the dietary fish oil level significantly affected the amounts of MUFA, PUFA, EPA + DHA, and n-3/n-6 ratio in all groups except for F6 and F2 groups held at 19 °C. The level of MUFA was significantly elevated in fish fed diet F2 + RV in comparison to fish fed diet F6 + RV for both holding temperatures ($p < 0.05$, Table IV- 6, indicated by m, n). The same applied for feeding groups F2 and F6 held at 23 °C. The reversed pattern was visible with regard to EPA + DHA and n-3/n-6 ration, with few exceptions. Dietary RV had almost no effect on the FA composition of the whole body homogenate with one exception. The amount of MUFA was significantly reduced in fish held at 19 °C and fed with diet F6 + RV in comparison to diet F6 ($p < 0.05$, Table IV- 6, indicated by A, B), but not for fish fed F2-based diets. This was also indicated by the evaluation of the statistical significance of effects, which showed that an interaction of dietary fish oil level and RV, but also an interaction of dietary fish oil level and holding temperature affected the MUFA content (Table IV- 5).

3.3.2. Liver tissue

The SFA, MUFA, and PUFA content and EPA/DHA ratio of liver tissue were only slightly affected by temperature and dietary treatment (Table IV- 7). Overall, the FA composition of the liver tissue seemed to be rather affected by the dietary treatment (fish oil and RV) than the holding temperature (Table IV- 5). The n-3/n-6 ratio was significantly affected by the dietary fish oil level, but not temperature. All fish fed the diets F6 and F6 + RV had significantly higher n-3/n-6 ratios compared to the corresponding F2 and F2 + RV feeding groups, respectively ($p < 0.05$, Table IV- 7, indicated by a, b and m, n). The amount of EPA + DHA was significantly affected by the interaction of all three factors, namely temperature, dietary fish oil, and RV-supplementation (Table IV- 5). Significantly higher EPA + DHA values were present in livers of fish held at 19 °C in comparison to the specimens held at 23 °C ($p < 0.05$, Table IV- 7, indicated by *). Additionally, fish at both holding temperatures fed with the diet F6 + RV had significantly higher EPA + DHA values in comparison to fish fed diet F2 + RV ($p < 0.05$, Table IV- 7, indicated by m, n). Fish fed diet F6 only had higher EPA + DHA values in comparison to the fish fed diet F2 when they were held at 23 °C ($p < 0.05$, Table IV- 7, indicated by a, b). Dietary RV significantly affected fish held at 19 °C, but not at 23 °C. Fish fed diet F6 + RV had significantly higher amount of EPA + DHA in comparison to fish fed diet F6 ($p < 0.05$, Table IV- 7, indicated by A, B). On the contrary, fish fed diet F2 + RV had significantly reduced values in comparison to fish fed diet F2 ($p < 0.05$, Table IV- 7, indicated by M, N).

Table IV- 6. Fatty acid composition (percentage of total fatty acid methyl esters (% FAMEs)) of whole body homogenates of gilthead sea bream at the end of the 8 week feeding trial held at two different temperatures (19 °C and 23 °C). F6 and F2 indicate the feeding with the basal diets containing 6% and 2% DM fish oil, respectively. +RV indicates feeding diets supplemented with 0.15% DM resveratrol.

[% FAMEs]	19 °C				23 °C				Comparison between 19 °C and 23 °C			
	F6	F2	F6 + RV	F2 + RV	F6	F2	F6 + RV	F2 + RV	F6	F2	F6 + RV	F2 + RV
14:0	2.8 ± 0.1 ^{a}	2.5 ± 0.1 ^{b}	2.7 ± 0.1 $^{(m)}$	2.5 ± 0.2 $^{(n)}$	2.8 ± 0.1 ^{a}	2.3 ± 0.1 ^{b}	2.8 ± 0.1 ^{m}	2.2 ± 0.2 ^{n}				*
16:0	17.2 ± 0.2	16.8 ± 0.2	17.0 ± 0.5	16.5 ± 0.1	18.9 ± 0.2	18.6 ± 0.5	18.7 ± 0.2	18.2 ± 0.4	***	***	***	***
18:0	4.4 ± 0.2	4.6 ± 0.1	4.6 ± 0.2	4.5 ± 0.0	5.1 ± 0.2 ^{A}	4.9 ± 0.1	4.7 ± 0.1 ^{B}	4.9 ± 0.1	***	*		**
Σ SFA ^{1}	**24.4 ± 0.4**	**23.9 ± 0.2**	**24.3 ± 0.6**	**23.5 ± 0.1**	**26.9 ± 0.4** ^{a}	**25.9 ± 0.5** ^{b}	**26.2 ± 0.3**	**25.4 ± 0.5**	***	***	***	***
14:1n-5	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0				
16:1n-7	4.4 ± 0.2 ^{a}	3.9 ± 0.1 ^{b}	4.2 ± 0.1	4.0 ± 0.3	4.5 ± 0.1 ^{a}	3.7 ± 0.2 ^{b}	4.5 ± 0.2 ^{m}	3.6 ± 0.2 ^{n}				
18:1n-7c	3.1 ± 0.1	3.0 ± 0.0	3.1 ± 0.1	3.0 ± 0.1	2.9 ± 0.1 ^{a}	2.6 ± 0.0 ^{b}	2.9 ± 0.0 ^{m}	2.7 ± 0.1 ^{n}	**	***	***	***
18:1n-9c	32.0 ± 0.7 ^{A}	32.8 ± 0.3	30.6 ± 1.0 ^{B,n}	33.1 ± 0.6 ^{m}	31.6 ± 0.4 ^{b}	35.5 ± 0.4 ^{a}	31.1 ± 0.3 ^{n}	35.3 ± 0.4 ^{m}		***		***
Σ MUFA ^{2}	**39.5 ± 0.9** ^{A}	**39.7 ± 0.2**	**38.1 ± 0.9** ^{B,n}	**40.2 ± 0.5** ^{m}	**39.2 ± 0.1** ^{b}	**41.8 ± 0.6** ^{a}	**38.5 ± 0.2** ^{n}	**41.6 ± 0.7** ^{m}		**		*
18:2n-6c	18.8 ± 0.6	19.5 ± 0.2	19.3 ± 0.5	19.3 ± 0.6	17.7 ± 0.3	18.6 ± 0.7	18.5 ± 0.2	18.9 ± 0.8	(*)			
18:3n-3	7.5 ± 0.3	7.6 ± 0.2	7.7 ± 0.2	7.4 ± 0.5	7.4 ± 0.0	8.0 ± 0.4	8.0 ± 0.2	8.2 ± 0.5				*
18:3n-6	0.9 ± 0.1 ^{B,b}	1.2 ± 0.0 ^{a}	1.1 ± 0.1 ^{A}	1.2 ± 0.1	0.8 ± 0.0	0.9 ± 0.1	0.8 ± 0.1 ^{n}	1.0 ± 0.1 ^{m}		***	***	**
20:5n-3	3.0 ± 0.1	2.7 ± 0.2	3.2 ± 0.3	2.9 ± 0.4	2.9 ± 0.1 ^{a}	1.7 ± 0.1 ^{b}	2.9 ± 0.1 ^{m}	1.7 ± 0.2 ^{n}		***		***
22:5n-3	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.1 ± 0.0 ^{a}	0.7 ± 0.0 ^{b}	1.1 ± 0.1 ^{m}	0.7 ± 0.1 ^{n}	*	***	**	***
22:6n-3	4.6 ± 0.2	4.2 ± 0.3	5.0 ± 0.4 ^{m}	4.4 ± 0.5 ^{n}	4.0 ± 0.0 ^{a}	2.5 ± 0.1 ^{b}	4.2 ± 0.2 ^{m}	2.5 ± 0.2 ^{n}	(*)	***	**	***
Σ PUFA ^{3}	**36.1 ± 1.1**	**36.4 ± 0.4**	**37.6 ± 1.5**	**36.3 ± 0.5**	**34.0 ± 0.3**	**32.3 ± 1.1**	**35.3 ± 0.2** ^{m}	**33.0 ± 1.0** ^{n}	*	***	*	**
EPA+DHA ^{4}	7.7 ± 0.3	6.9 ± 0.5	8.2 ± 0.7 $^{(m)}$	7.3 ± 0.8 $^{(n)}$	6.9 ± 0.1 ^{a}	4.1 ± 0.1 ^{b}	7.0 ± 0.3 ^{m}	4.3 ± 0.3 ^{n}		***	*	***
EPA/DHA ^{4}	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0				
n-3/n-6 ^{5}	0.8 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.0				

^{1} ∑ SFA is the sum of saturated fatty acids; ^{2} ∑ MUFA is the sum of monounsaturated fatty acids; ^{3} ∑ PUFA is the sum of n-3 and n-6 polyunsaturated fatty acids; ^{4} EPA: Eicosapentaenoic acid (20:5n-3) and DHA: Docosahexaenoic acid (22:6n-3); ^{5} n-3/n-6 is the ratio between all n-3 and all n-6 fatty acids measured

Values (mean ± SD, n=3) with different superscript letters and different types of letters within one temperature treatment differ with *p*-values < 0.05 based on ANOVA as described in Materials & methods. Superscript letters indicate the output of tests based on comparisons of fish oil level within one supplement group (a, b: F6 vs. F2; m, n: F6 + RV vs. F2 + RV) and effect of supplementation within one fish oil level (A, B: F6 vs. F6 + RV), separated by temperature. The statistical output of test based on the effect of the different holding temperatures analyzed within one feeding group are indicated in separate columns using * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. All designations in brackets indicate a tendency towards a difference based on $p < 0.1$.

Table IV- 7. Fatty acid composition (percentage of total fatty acid methyl esters (% FAMEs)) of liver tissue of gilthead sea bream at the end of the 8 week feeding trial held at two different temperatures (19 °C and 23 °C). F6 and F2 indicate the feeding with the basal diets containing 6% and 2% DM fish oil, respectively. +RV indicates feeding diets supplemented with 0.15% DM resveratrol.

[% FAMEs]	19 °C				23 °C				Comparison between 19 °C and 23 °C			
	F6	F2	F6 + RV	F2 + RV	F6	F2	F6 + RV	F2 + RV	F6	F2	F6 + RV	F2 + RV
14:0	1.7 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	2.2 ± 0.3	2.2 ± 0.2	2.1 ± 0.2	2.2 ± 0.1	**	**	**	***
16:0	15.5 ± 0.6	14.1 ± 0.8	14.4 ± 1.2	14.4 ± 0.9	19.4 ± 0.8	18.4 ± 0.4	20.1 ± 0.6	19.4 ± 0.2	***	***	***	***
18:0	6.4 ± 0.5	6.4 ± 0.4	6.9 ± 0.8	6.3 ± 0.6	7.8 ± 0.0	7.6 ± 0.2	8.0 ± 0.0	7.5 ± 0.6	*	*	*	*
Σ SFA [1]	**23.6 ± 1.0**	**22.2 ± 1.2**	**22.9 ± 2.0**	**22.2 ± 1.4**	**29.4 ± 0.9**	**28.2 ± 0.7**	**30.3 ± 0.8**	**29.1 ± 0.6**	***	***	***	***
14:1n-5	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0				
16:1n-7	3.8 ± 0.1 ^{a}	3.1± 0.1 ^{b}	3.4 ± 0.0 ^{m}	2.9 ± 0.3 ^{n}	3.8 ± 0.1 ^{a}	3.2 ± 0.2 ^{b}	4.0 ± 0.1 ^{m}	3.5 ± 0.3 ^{n}			**	**
18:1n-7c	3.0 ± 0.1	2.9 ± 0.0	3.0 ± 0.1	2.9 ± 0.0	2.8 ± 0.1	2.6 ± 0.1	2.9 ± 0.1 ^{m}	2.6 ± 0.2 ^{n}	(*)	*		**
18:1n-9c	39.0 ± 0.7	41.1 ± 0.5	37.8 ± 1.8 ^{n}	43.4 ± 1.5 ^{m}	40.3 ± 1.9	41.5 ± 0.3	39.6 ± 0.7 ^{n}	42.9 ± 0.8 ^{m}				
Σ MUFA [2]	**45.8 ± 0.6**	**47.1 ± 0.4**	**44.3 ± 1.7 ^{n}**	**49.2 ± 1.7 ^{m}**	**47.0 ± 1.9**	**47.4 ± 0.4**	**46.5 ± 0.6 $^{(n)}$**	**49.1 ± 0.4 $^{(m)}$**				
18:2n-6c	16.2 ± 0.8	16.3 ± 1.2	16.5 ± 1.7	16.2 ± 0.7	11.9 ± 0.5	13.1 ± 0.6	11.7 ± 0.5	12.0 ± 0.7	***	**	***	***
18:3n-3	5.9 ± 0.5	5.3 ± 0.5	5.5 ± 0.9	5.5 ± 0.5	4.2 ± 0.3	4.3 ± 0.4	4.0 ± 0.2	3.9 ± 0.5	**		**	**
18:3n-6	2.2 ± 0.2 ^{b}	3.7 ± 0.9 ^{a}	3.0 ± 0.6	3.3 ± 0.7	1.9 ± 0.1 ^{b}	3.5 ± 0.6 ^{a}	1.9 ± 0.3	2.9 ± 1.1				
20:5n-3	2.0 ± 0.1 ^{B}	1.7 ± 0.4 ^{M}	2.5 ± 0.2 ^{A,m}	1.1 ± 0.2 ^{N,n}	1.8 ± 0.1 ^{a}	1.1 ± 0.1 ^{b}	1.8 ± 0.1 ^{m}	0.9 ± 0.1 ^{n}		*	**	
22:5n-3	0.7 ± 0.0	0.5 ± 0.1	0.7 ± 0.1 ^{m}	0.4 ± 0.1 ^{n}	0.6 ± 0.1 ^{a}	0.3 ± 0.0 ^{b}	0.6 ± 0.0 ^{m}	0.3 ± 0.1 ^{n}		**		
22:6n-3	3.6 ± 0.3 ^{B}	3.2 ± 0.8 ^{M}	4.6 ± 0.4 ^{A,m}	2.1 ± 0.5 ^{N,n}	3.2 ± 0.2 ^{a}	2.1 ± 0.3 ^{b}	3.2 ± 0.2 ^{m}	1.9 ± 0.2 ^{n}		*	**	
Σ PUFA [3]	**30.5 ± 1.6**	**30.7 ± 1.4**	**32.9 ± 3.6 ^{m}**	**28.6 ± 0.7 ^{n}**	**23.6 ± 1.1**	**24.4 ± 1.1**	**23.2 ± 0.3**	**21.9 ± 1.0**	***	***	***	***
EPA+DHA [4]	5.6 ± 0.4 ^{B}	4.9 ± 1.2 ^{M}	7.1 ± 0.6 ^{A,m}	3.2 ± 0.7 ^{N,n}	5.0 ± 0.3 ^{a}	3.2 ± 0.5 ^{b}	5.0 ± 0.3 ^{m}	2.8 ± 0.3 ^{n}		*	**	
EPA/DHA [4]	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0				**
n 3/n 6 [5]	0.7 ± 0.0 ^{a}	0.5 ± 0.1 ^{b}	0.7 ± 0.0 ^{m}	0.5 ± 0.0 ^{n}	0.7 ± 0.0 ^{a}	0.5 ± 0.0 ^{b}	0.7 ± 0.0 ^{m}	0.5 ± 0.1 ^{n}				

[1] ∑ SFA is the sum of saturated fatty acids; [2] ∑ MUFA is the sum of monounsaturated fatty acids; [3] ∑ PUFA is the sum of n-3 and n-6 polyunsaturated fatty acids; [4] EPA: Eicosapentaenoic acid (20:5n-3) and DHA: Docosahexaenoic acid (22:6n-3); [5] n-3/n-6 is the ratio between all n-3 and all n-6 fatty acids measured

Values (mean ± SD, n=3) with different superscript letters and different types of letters within one temperature treatment differ with *p*-values < 0.05 based on ANOVA as described in Materials & methods. Superscript letters indicate the output of tests based on comparisons of fish oil level within one supplement group (a, b: F6 vs. F2; m, n: F6 + RV vs. F2 + RV) and effect of supplementation within one fish oil level (A, B: F6 vs. F6 + RV; M, N: F2 vs. F2 + RV), separated by temperature. The statistical output of test based on the effect of the different holding temperatures analyzed within one feeding group are indicated in separate columns using * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. All designations in brackets indicate a tendency towards a difference based on $p < 0.1$.

Table IV- 8. Fatty acid composition (percentage of total fatty acid methyl esters (% FAMEs)) of fillet tissue of gilthead sea bream at the end of the 8 week feeding trial held at two different temperatures (19 °C and 23 °C). F6 and F2 indicate the feeding with the basal diets containing 6% and 2% DM fish oil, respectively. +RV indicates feeding diets supplemented with 0.15% DM resveratrol.

[% FAMEs]	19 °C				23 °C				Comparison between 19 °C and 23 °C			
	F6	F2	F6 + RV	F2 + RV	F6	F2	F6 + RV	F2 + RV	F6	F2	F6 + RV	F2 + RV
14:0	2.5 ± 0.2 ^{a}	2.2 ± 0.2 ^{b}	2.7 ± 0.1 ^{m}	2.1 ± 0.1 ^{n}	2.6 ± 0.1 ^{a}	2.1 ± 0.0 ^{b}	2.4 ± 0.2 ^{m}	2.0 ± 0.2 ^{n}				
16:0	17.9 ± 0.1 ^{a}	17.2 ± 0.2 ^{b}	17.5 ± 0.1	17.1 ± 0.2	19.2 ± 0.3	19.5 ± 0.3	19.5 ± 0.4	19.2 ± 0.3	***	***	***	***
18:0	4.3 ± 0.2	4.4 ± 0.1	4.4 ± 0.0	4.4 ± 0.1	4.9 ± 0.2	4.9 ± 0.2	4.8 ± 0.2	4.9 ± 0.1	***	**	**	***
Σ SFA ^{1}	**24.8 ± 0.2** ^{a}	**23.8 ± 0.3** ^{b}	**24.5 ± 0.2** ^{m}	**23.6 ± 0.2** ^{n}	**26.7 ± 0.3**	**26.5 ± 0.4**	**26.8 ± 0.5**	**26.2 ± 0.3**	***	***	***	***
14:1n-5	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0				
16:1n-7	4.0 ± 0.3 ^{a}	3.6 ± 0.3 ^{b}	4.0 ± 0.1 ^{m}	3.4 ± 0.1 ^{n}	4.2 ± 0.1 ^{a}	3.6 ± 0.1 ^{b}	4.1 ± 0.1 ^{m}	3.5 ± 0.2 ^{n}				
18:1n-7c	2.9 ± 0.0	2.8 ± 0.1	3.0 ± 0.1 ^{m}	2.8 ± 0.0 ^{n}	2.7 ± 0.1 ^{a}	2.4 ± 0.1 ^{b}	2.7 ± 0.0 ^{m}	2.5 ± 0.0 ^{n}	***	***	***	***
18:1n-9c	31.2 ± 0.5 ^{b}	33.0 ± 0.3 ^{a}	30.3 ± 0.9 ^{n}	32.6 ± 0.7 ^{m}	31.1 ± 0.2 ^{b}	34.0 ± 0.7 ^{a}	31.2 ± 0.4 ^{n}	33.8 ± 1.0 ^{m}				
Σ MUFA ^{2}	**38.2 ± 0.1**	**39.4 ± 0.3**	**37.4 ± 0.9** $^{(n)}$	**38.8 ± 0.6** $^{(m)}$	**38.0 ± 0.2** ^{b}	**40.0 ± 0.7** ^{a}	**38.1 ± 0.4** ^{n}	**39.8 ± 1.2** ^{m}				
18:2n-6c	19.7 ± 0.2	20.1 ± 0.5	19.7 ± 0.2 ^{n}	20.6 ± 0.2 ^{m}	18.5 ± 0.1	19.1 ± 0.2	18.5 ± 0.4 ^{n}	19.4 ± 0.5 ^{m}	**	**	**	**
18:3n-3	8.3 ± 0.1	8.3 ± 0.2	8.2 ± 0.2	8.4 ± 0.2	7.8 ± 0.1	8.2 ± 0.2	8.0 ± 0.3	8.2 ± 0.2	**			
18:3n-6	0.8 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	1.0 ± 0.1	0.7 ± 0.0	0.7 ± 0.1	0.6 ± 0.1 ^{n}	0.8 ± 0.1 ^{m}		***	***	***
20:5n-3	2.8 ± 0.1	2.5 ± 0.2	3.2 ± 0.3 ^{m}	2.5 ± 0.2 ^{n}	2.7 ± 0.0 ^{a}	1.8 ± 0.3 ^{b}	2.6 ± 0.0 ^{m}	1.9 ± 0.2 ^{n}		**	*	**
22:5n-3	1.2 ± 0.0 $^{(B)}$	1.1 ± 0.1	1.4 ± 0.1 $^{(A),m}$	1.1 ± 0.1 ^{n}	1.2 ± 0.1 ^{a}	0.8 ± 0.1 ^{b}	1.1 ± 0.0 ^{m}	0.8 ± 0.1 ^{n}		**	**	***
22:6n-3	4.3 ± 0.1	3.9 ± 0.3	4.8 ± 0.5 ^{m}	4.0 ± 0.3 ^{n}	4.4 ± 0.0 ^{a}	3.0 ± 0.4 ^{b}	4.3 ± 0.2 ^{m}	3.1 ± 0.6 ^{n}		*		*
Σ PUFA ^{3}	**37.0 ± 0.2**	**36.8 ± 0.5**	**38.1 ± 0.7**	**37.7 ± 0.6**	**35.3 ± 0.3** ^{a}	**33.5 ± 0.9** ^{b}	**35.2 ± 0.7**	**34.0 ± 1.4**	*	***	***	***
EPA+DHA ^{4}	7.1 ± 0.2	6.3 ± 0.4	8.0 ± 0.7 ^{m}	6.6 ± 0.5 ^{n}	7.1 ± 0.1 ^{a}	4.8 ± 0.7 ^{b}	7.0 ± 0.2 ^{m}	4.9 ± 0.8 ^{n}		**		**
EPA/DHA ^{4}	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0				
n-3/n-6 ^{5}	0.8 ± 0.0	0.8 ± 0.0	0.9 ± 0.0 ^{m}	0.7 ± 0.0 ^{n}	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.0				

^{1} ∑ SFA is the sum of saturated fatty acids; ^{2} ∑ MUFA is the sum of monounsaturated fatty acids; ^{3} ∑ PUFA is the sum of n-3 and n-6 polyunsaturated fatty acids; ^{4} EPA: Eicosapentaenoic acid (20:5n-3) and DHA: Docosahexaenoic acid (22:6n-3); ^{5} n-3/n-6 is the ratio between all n-3 and all n-6 fatty acids measured

Values (mean ± SD, n=3) with different superscript letters and different types of letters within one temperature treatment differ with *p*-values < 0.05 based on ANOVA as described in Materials & methods. Superscript letters indicate the output of tests based on comparisons of fish oil level within one supplement group (a, b: F6 vs. F2; m, n: F6 + RV vs. F2 + RV) and effect of supplementation within one fish oil level (A, B: F6 vs. F6 + RV), separated by temperature. The statistical output of test based on the effect of the different holding temperatures analyzed within one feeding group are indicated in separate columns using * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. All designations in brackets indicate a tendency towards a difference based on $p < 0.1$.

3.3.3. Fillet tissue

The FA composition of fillet tissue was affected by the interaction of dietary fish oil and temperature, except for MUFA which was only affected by the dietary fish oil level (Table IV- 5). Overall, the amount of SFA was significantly increased in fish held at 23 °C in comparison to fish held at 19 °C ($p < 0.05$, Table IV- 8, indicated by *). The opposite applied for PUFA (all diets) and for EPA + DHA and n-3/n-6 (only for F2-based diets). Whereas the amount of SFA and the n-3/n-6 ratio were rather reduced when F2-based diets were fed, the amount of MUFAs was increased (Table IV- 8). The amount of EPA + DHA was significantly higher in fish held at both temperatures and fed the diet F6 + RV in comparison to the diet F2 + RV ($p < 0.05$, Table IV- 8, indicated by m, n). The same applied for fish fed diet F6 in comparison to diet F2, but only at 23 °C holding temperature ($p < 0.05$, Table IV- 8, indicated by a, b).

3.4. The gene expression in liver was predominantly affected by dietary fish oil level

The mRNA steady state levels of selected genes were only measured in livers of sea bream held at the lower temperature (19 °C) (Table IV- 6). The mRNA levels of Δ6-D, PPARα, ECH, and CEL were affected by the dietary treatment (Figure IV- 1). The reduction of the dietary fish oil level from 6% DM (diet F6) to 2% DM (diet F2) significantly elevated the mRNA steady state levels of all 4 genes ($p < 0.05$, Figure IV- 1, indicated by a, b). The mRNA steady states of Δ6-D and PPARα were also significantly elevated when the dietary fish oil level was reduced in the RV-supplemented diets (dietary groups F6 + RV and F2 + RV, $p < 0.05$, Figure IV- 1 (a) and (b), indicated by m, n). The fish fed the diet F6 + RV had significantly elevated hepatic mRNA steady state levels of Δ6-D in comparison to fish fed the basal diet F6 ($p < 0.05$, Figure IV- 1 (a), indicated by *). Furthermore, the mRNA level of PPARα was significantly reduced in the livers of fish fed the diet F2 + RV in comparison to F2 ($p < 0.05$, Figure IV- 1 (b), indicated by *).

4. Discussion

Throughout the experimental period of eight weeks, the juvenile sea bream of all groups exhibited good growth and performance (Table IV- 4). The diet formulation with a crude lipid content of 14.5% DM and strictly reduced fish meal (5% DM) and fish oil (2% DM) content did not impair the fish growth and performance. The ability of sea bream to cope with diet formulations reduced in marine ingredients has previously been shown, but extruded diets with higher crude lipid content (approx. 25% DM) and higher fish meal (36 – 38% DM) or fish meal and fish concentrate (15% and 5% DM, respectively) and fish oil content (7 – 3.5% DM) had been used (Benedito-Palos et al., 2009, 2008, Izquierdo et al., 2005, 2003; Menoyo et al., 2004).

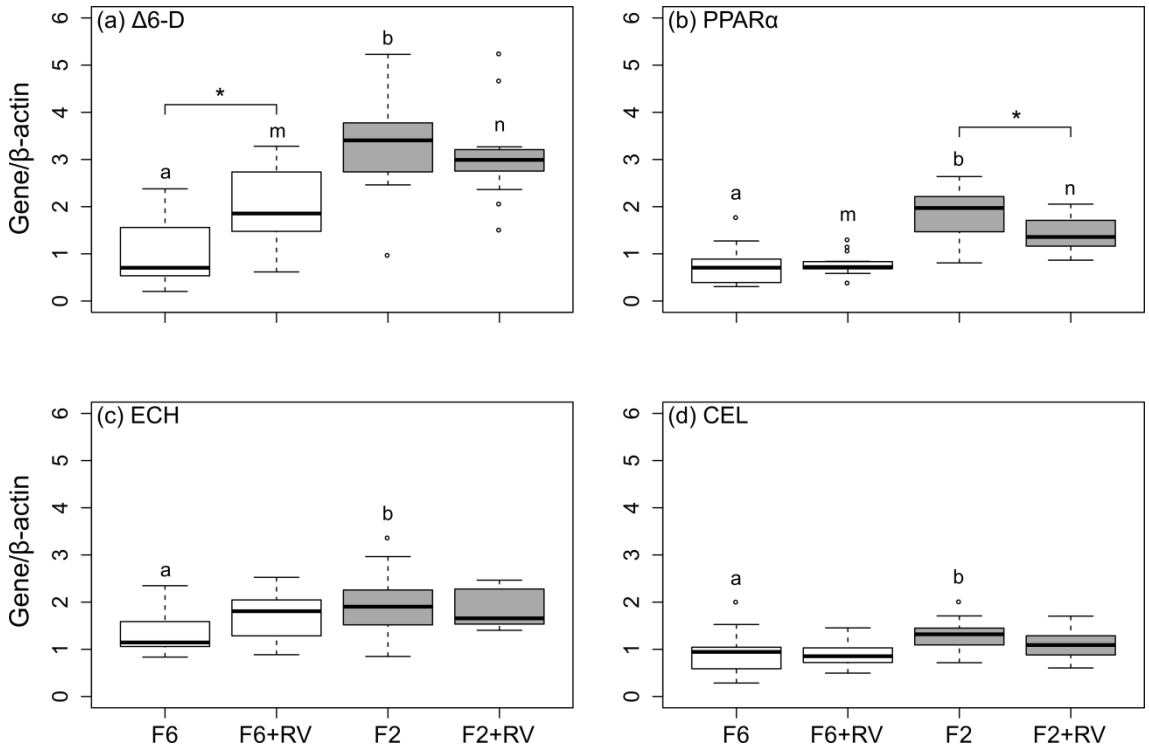

Figure IV- 1. Hepatic mRNA steady state levels in livers of gilthead sea bream held at 19 °C and fed different diets over duration of eight weeks. The fish were fed with the basal diets F6 and F2 (6% DM and 2% DM fish oil, respectively) and the experimental diets supplemented with 0.15% DM resveratrol (+RV diets). **(a)** Δ6-D: Δ6-desaturase, **(b)** PPARα: peroxisome proliferator-activated receptor α, **(c)** ECH: enoyl-CoA hydratase, and **(d)** CEL: carboxyl ester lipase were measured in the liver of fish using qRT-PCR and were normalized to the housekeeping gene ß-actin. Boxes represent values (n=15) between the 25 and 75 percentiles, whiskers indicate 1.5 SD; the solid line indicates the median; circles represent values above and below SD. Significant differences ($p < 0.05$) were analyzed using multiple contrast tests for relative effects. Tests were based on comparisons of dietary fish oil level within one supplement group (no supplement: a, b; +RV: m, n) or supplement type within one dietary fish oil level (indicated by *).

The combined exchange of fish meal and fish oil (15% DM and 5% DM, respectively (Fountoulaki et al., 2009) and 12.5% DM and below 4.4% DM, respectively, (Houston et al., 2017)) with plant ingredients had previously led to growth depression of gilthead sea bream. It is commonly accepted that sea bream need minimum amounts of n-3 LC-PUFAs and a minimum EPA + DHA amount of 0.9% of dry diet for good growth and performance (Kalogeropoulos et al., 1992; NRC, 2011). It is noteworthy that the sea bream in this trial had no growth and performance depression with the F2-based diets, although the amount of EPA and DHA was only 0.37% of dry diet and thus below the generally accepted recommendations.

The distinctive impairment of DFI, SGR, FBW, and FCF at 19 °C represents normal adaptions of the sea bream to lower temperatures and is in accordance with previous findings (Couto et al., 2008; Tort et al., 2004). The SGR (1.3 – 2.4% d^{-1}) was within the range or even better than previously reported by other studies with sea bream of

comparable size held at similar water temperatures (1.2 – 1.6% d^{-1} (Pereira and Oliva-Teles, 2003) and 1.8 – 1.9% d^{-1} (Izquierdo et al., 2003)). The DFI (3.2 – 4.3% d^{-1}) and FCR (1.9 – 2.3) were higher than reported in the aforementioned studies. Additionally, the nutrient utilization parameters PER (approx. 1.1) and PPV (approx. 16.4%) were at the lower margin as previously reported (PER: 1.02 – 1.51 (Fountoulaki et al., 2009; Pereira and Oliva-Teles, 2003) and PPV: 17.7 – 23.6% (Pereira and Oliva-Teles, 2003). The diets low in fish meal (5% DM) and high in plant ingredients might have affected the nutrient utilization and thus PER, PPV, and FCR. Furthermore, the DFI was calculated based on weighed daily feed ratios and it cannot be excluded that feed was lost due to the water current. Thus these values might overestimate the actual DFI and in return might result in an underestimation of the actual FCR, PER, and PPV. The effect of reduced dietary fish oil content on the HSI of sea bream was most pronounced in fish held at the lower water temperature and fed RV-supplemented diets (F2 + RV vs. F6 + RV). Generally, the HSI of sea bream increases when the amount of dietary vegetable oil is increased (Fountoulaki et al., 2009; Menoyo et al., 2004), which was only partly visible within this study.

The difference in holding temperature and dietary fish oil level partly affected the whole body nutrient composition of the sea bream (Table IV- 4). The whole body crude lipid content was increased in sea bream held at the higher water temperature. A slight but non-significant increase in crude lipid at a higher water temperature had been previously observed in sea bass (Person-Le Ruyet et al., 2004; Skalli et al., 2006). In the present study, the increased DFI of groups held at 23 °C might have contributed to this effect. Furthermore, a reduction in whole body crude lipid conditioned by reduced dietary fish oil inclusion as observed in this study had been previously observed in sea bream (Kalogeropoulos et al., 1992) and might be a result of qualitative differences in the used dietary oil sources. Dietary supplementation of RV to the F6 diet (F6 + RV) led to reduced whole body crude lipid and gross energy in tendency. Similar had been observed in studies from our group with rainbow trout fed RV-supplemented diets, which exhibited reduced whole body crude lipid in tendency (Torno et al. unpublished results, see **chapter II**). Possible explanations might be a reduced lipid digestibility as observed in the rainbow trout trial, or metabolic changes, amongst others, as extensively investigated in obesity and body weight management studies (Rupasinghe et al., 2016).

Irrespective of the dietary treatment and temperature challenge, all sea bream had visibly increased amounts of MUFAs in their tissues compared to the diets. The predominant group of FAs in the fish tissues was MUFAs followed by PUFAs, whereas in all diets it was PUFAs. The amount of MUFAs can be mainly attributed to the FA 18:1n-9c (Tables IV- 6, 7, and 8). Furthermore, it seemed that the sea bream readily took up all EPA and DHA offered *via* the diet, whereas less 18:3n-3 (ALA) and 18:2n-6 were taken up

from the diet (Tables IV- 6, 7, and 8). Previous studies had reported, that the FA composition of sea bream tissue greatly reflects the FA composition of the diet (Houston et al., 2017; Izquierdo et al., 2003; Menoyo et al., 2004). The high MUFA content of the sea bream from this trial might be mediated by the predominantly vegetable diet formulation, since an increase in MUFAs is often associated with an increase in dietary plant raw materials and oils (Houston et al., 2017; Izquierdo et al., 2005, 2003). It is hypothesized, that the high amount of carbohydrates in diets with plant proteins functioned as source for FA and increased amounts of 18:1n-9. Investigation of this overall effect was beyond the scope of this study and proof cannot be given unless further research is conducted. Nevertheless, an increase of MUFAs in sea bream (fillet tissue and whole body homogenate) fed the F2-based diets in comparison to the F6-based diets was visible in this study. Furthermore, a higher MUFA to SFA ratio was present in the fish held at 19 °C and might be an adaptation to the lower temperature in order to maintain physiological and cellular functions (Sargent et al., 2002; Wodtke and Cossins, 1991).

The evaluation of the statistical significance of effects demonstrated, that the FA composition of whole body and fillet tissue was predominantly affected by the interaction of the dietary fish oil level and the holding temperature (Table IV- 5). This is particularly prominent with regard to PUFA and EPA + DHA content. A decrease in PUFA and EPA + DHA content due to the reduced dietary fish oil level was mainly present when fish were held at the higher water temperature. The temperature-mediated difference in feed intake and growth might have caused these effects in the first place. If we assume that the change in the tissue FA profile follows a simple dilution model (dilution of tissue FAs by dietary FAs), a fast growth with high tissue FA turnover would accelerate the dilution process (Benedito-Palos et al., 2009). Furthermore, Izquierdo et al. (2005) demonstrated that the greatest change in FA profile happens within 60 – 100 days before a steady state is reached. In the current experiment, a reduction of EPA + DHA had taken place from the beginning of the experiment to the end after 56 days (Table IV- 5). During this time, fish held at 23 °C tripled their weight, whereas fish held at 19 °C doubled their weight. Simultaneously, the final whole body and fillet tissue EPA + DHA content was lower in fish held at 23 °C. Within the dietary treatment groups held at 23 °C, the fish fed the F2-based diets with extremely low EPA + DHA content (0.37% of dry diet) had significantly lower final EPA + DHA levels compared to those fed the F6-based diets. Thus, the dilution of the previous tissue EPA + DHA content was more severe in fish with faster growth and fed extremely low amounts of EPA + DHA. Other environmentally triggered mechanisms affecting the FA composition of tissues in order to maintain functionality of physiological processes (Sargent et al., 2002) might have occurred simultaneously but cannot be clearly determined.

The response of the FA composition to the interaction of dietary fish oil level and RV-supplementation in the liver tissue is of great interest (Tables IV- 5 and 6). The EPA + DHA content in livers of sea bream held at 19 °C was significantly altered caused by the dietary administration of RV, but differently at the two dietary fish oil levels. The supplementation of RV to the diet with 6% DM fish oil led to a significant increase in EPA + DHA content. These LC-PUFA increasing effects of RV had been previously reported for rat hepatocytes (Momchilova et al., 2014), zebra fish embryos (Caro et al., 2017), and rainbow trout (Torno et al., 2017). On the contrary, RV supplementation to the diet wit 2% DM fish oil led to a decrease in EPA + DHA content in the sea bream livers. It is not fully clear, why RV acted differently in the various dietary treatments. In our previous study with rainbow trout, we already observed that RV affects the tissue PUFA levels dependent on the dietary fish oil level (Torno et al., 2017). In addition, Ran et al. (2017) described a bi-directional interaction of RV with the lipid metabolism of zebra fish. In Atlantic salmon fed graded levels of RV-supplemented diets, RV did not positively affect the FA composition but impaired feed intake and thus eventually growth (Menoyo et al. 2018). Whether the differences in the response of the fish to dietary RV can be associated with the difference in species, the difference in lifestyle (freshwater vs. marine), or other factors cannot be concluded from such few studies yet.

The mRNA steady state levels of the Δ6-D gene and genes involved in the FA metabolism and β-oxidation were significantly affected by the dietary fish oil level and also partly by dietary RV-supplementation (Figure IV- 1). A diet induced up-regulation of the mRNA expression and enzyme activity of the Δ6-D, a key enzyme in FA synthesis, had been observed in marine fish fed PUFA-deficient diets, for example sea bream (Izquierdo et al., 2008; Seiliez et al., 2003) and sea bass (González-Rovira et al., 2009). With the results obtained in this study, we can support the previously observed upregulated mRNA expression of the Δ6-D when sea bream are fed diets low in fish oil, but cannot draw conclusions on the abundance or activity of the Δ6-D enzyme. Furthermore, the increase in PPARα mRNA steady state levels caused by a decrease in dietary fish oil level could have been mediated by the dietary PUFAs, which are substrates for PPARs (Kliewer et al., 1997). It has been shown that the nutritional status of fish influences the mRNA expression of PPARα (Leaver et al., 2005), a transcription factor involved in many aspects of the hepatic lipid metabolism including FA uptake, FA activation, and FA oxidation (Rakhshandehroo et al., 2010). In this study, the mRNA steady state levels of ECH and CEL was increased at the very low dietary fish oil level. A dietary induced regulation of the hepatic mRNA expression of ECH and CEL had been suggested previously for sea bream (Benedito-Palos et al., 2014; Pérez-Sánchez et al., 2013) and sea bass (Rimoldi et al., 2016). An interaction between dietary fish oil and PUFA content and expression of ECH and CEL seems likely, since ECH is involved in the mitochondrial β-oxidation of FA

(Benedito-Palos et al., 2014) and CEL is involved in lipid and FA uptake, transport, and metabolism (Hui and Howles, 2002).

In this study, the RV-mediated modification of EPA + DHA levels in livers of sea bream can be (partly) explained by differences in the mRNA steady state levels of Δ6-D and PPARα (Figure IV- 1). The Δ6-D mRNA steady state level was significantly elevated in livers of fish fed diet F6+RV in comparison to F6. This can be regarded as an explanation for the simultaneously increased EPA + DHA content in the fish livers. This is supported by previous findings in rat hepatocytes, where RV interacted with the Δ6- and Δ5-desaturases, thus increasing the LC-PUFA content (Momchilova et al., 2014). The decreased PPARα mRNA steady state levels in fish fed diet F2 + RV in comparison to F2 might be a possible explanation for the concomitant decrease in EPA + DHA content in the fish from those feeding groups. The RV-mediated reduced steady state level of the PPARα-gene is controversial as compared to previous studies in which RV was described as PPAR activator (reviewed by Nakata et al. (2012)), or where RV had no effect on PPARα expression (Caro et al., 2017; Torno et al., 2017). We assume that other mechanisms and molecular pathways might have been modified by RV and influenced the FA composition in sea bream liver tissue (Ajmo et al., 2008; Frémont et al., 1999; Ran et al., 2017; Rupasinghe et al., 2016). Nevertheless, with the results obtained in this study, we can expand the knowledge concerning the genetic capacity of sea bream to perform endogenous FA synthesis. The dietary oil source not only influences the tissue LC-PUFA levels, but affects genes involved in FA synthesis and metabolism. The role of RV in the nutritional regulation of the mRNA steady state levels of Δ6-D and PPARα still needs to be clarified. From this study it cannot be fully concluded whether or not RV stimulated FA synthesis. No enzyme activities were measured and no positive FA retention could be observed, thus indicating a loss of tissue PUFAs and especially EPA + DHA levels throughout the experimental trial.

5. Conclusion

Within this study we fed juvenile sea bream with diets very low in fish meal (5% DM) and fish oil (2% DM) without affecting the growth, performance, and nutrient utilization parameters. Nevertheless, a decrease in whole body crude lipid and gross energy content was visible in fish fed the low-fish oil diets. Furthermore, the dietary treatments affected the FA composition and resulted in increased MUFA content in fish fed the low-fish oil diets. The amounts of PUFAs and EPA + DHA in whole body, fillet, and liver tissue were greatly affected by the dietary fish oil level and exhibited signs of a wash-out effect throughout the experimental period. The dietary induced reduction of EPA + DHA was most pronounced in fish with fast growth and fed the diets with extremely low EPA + DHA

content (0.37% of dry diet). It was visible, that the temperature challenge and thus increased growth at the higher holding temperature affected the body nutrient and FA composition. The dietary administration of RV only affected the EPA + DHA content in livers of sea bream held at the lower water temperature. The RV-mediated change in the hepatic mRNA steady state levels of the Δ6-D and PPARα genes can be a possible explanation for the change in liver EPA + DHA content. Overall, the use of RV in low-fish oil diets had neither beneficial, nor detrimental effects on growth, performance, body composition, and whole body FA profile of juvenile sea bream. Dietary RV modulated the liver EPA and DHA content and mRNA steady state levels of genes involved in FA metabolism. Further research is necessary to clarify whether RV might be a possible fish oil complement in marine fish nutrition.

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GENERAL DISCUSSION

This thesis aimed to investigate the suitability of the two bioactive substances resveratrol (stilbene) and genistein (phytoestrogen) as additives for fish oil replacement in aquafeeds for freshwater and marine fish. The major part of the thesis focused on the use of diets enriched with the bioactives in rainbow trout (*Oncorhynchus mykiss*) nutrition. The investigated effects of resveratrol and genistein on the endogenous fatty acid biosynthesis (**chapters I and II**) were supported by investigations of the growth and performance parameters (**chapter II**) as well as nutrient digestibility and histopathological examinations (**chapter III**). The last chapter comprised investigations of the bioactive resveratrol on the endogenous fatty acid biosynthesis, growth, and performance parameters of gilthead sea bream (*Sparus aurata*) (**chapter IV**). The following sections will discuss the suitability of resveratrol and genistein as additives for fish oil replacement in the context of the experimental results obtained herein.

Fish oil replacement in aquafeeds

Numerous review articles and book chapters deal with the issue of fish oil replacement in aquafeeds, highlighting the importance of this topic and the difficulty to find a suitable alternative for adequate fish nutrition (e.g. Nasopoulou and Zabetakis, 2012; Tocher, 2015; Turchini et al., 2011, 2009). The most important task includes finding a new, sustainable, and suitable source of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Potential fish oil replacements that meet this scope need to be investigated for their effects on growth and performance parameters of fish. This is often intensified by investigations of metabolic and physiological aspects, followed by investigations of the fish health status. Finally, the quality and nutritional value of products from fish fed potential fish oil replacements need to be monitored. Novel feed ingredients and potential fish oil replacements should be carefully examined with regard to the aforementioned aspects.

The lipid quality of aquafeeds is changed when fish oil is replaced by plant oils, since plant oils lack EPA and DHA but instead contain more omega-6 fatty acids and shorter chained fatty acids. Many studies observed no impairment of growth and performance of fish fed diets rich in plant oils but detected decreased lipid quality in terms of reduced EPA and DHA levels. This was shown in short term experiments (Benedito-Palos et al., 2010; Izquierdo et al., 2005) as well as whole production cycles (Lazzarotto et al., 2018, 2015; Mourente and Bell, 2006; Sissener et al., 2016; Thanuthong et al., 2011). This practice of fish oil replacement has already affected the overall quality of farmed fish to a great

extent. Some fish species, e.g. salmon and sea bream, have distinctive inferior omega-3 (n-3) highly unsaturated fatty acid (HUFA) contents in comparison to wild counterparts or farmed fish from previous years (Sprague et al., 2016; Vasconi et al., 2017). Tocher (2015) uses a three-level approach to explain required n-3 HUFA levels in aquafeeds: (1) the first n-3 HUFA level is a minimum level to satisfy the fish requirements for physiological and cellular functions and is around 1% of the diet, (2) the second level is a bit higher and would be sufficient to ensure maximum growth and health, (3) and the third level is not based on the requirements of the fish, but the consumers demand for high quality products. The commonly accepted requirement levels given by the National Research Council (NRC, 2011) are oriented on studies evaluating the minimum requirements of fish (level 1) and levels required for good growth, performance, and health (level 2). The dietary formulations used in this thesis are based on the EPA and DHA requirements of the respective species given by the NRC (2011). Thus, the diets for sea bream (EPA + DHA: 0.37 – 0.92% of dry matter of diet) had sufficient n-3 HUFAs to meet minimum requirements but revealed impaired tissue lipid quality of those fish fed the low-fish oil diets (**chapter IV**). The diets for rainbow trout (minimum EPA + DHA: 0.30% dry matter of diet) did neither impair fish growth nor fatty acid composition. Rainbow trout can satisfy their minimum n-3 HUFA requirements by elongation and desaturation of shorter chained fatty acids like α-linolenic acid (ALA,18:3n-3), averting a significantly decreased lipid quality (**chapters I and II**).

Nevertheless, fish oil replacement should not only aim at minimum requirement levels of essential n-3 HUFAs for the fish species, if the consumer is to be satisfied. In order to produce fish rich in n-3 HUFAs and simultaneously improve the sustainability of aquafeeds and aquaculture, attempts focusing on the efficiency of fish to utilize plant oils should be developed further. One possibility, among others, is to make use of the genetic capacity of fish, which enables them to perform the fatty acid synthesis. The whole potential of the *in vivo* fatty acid biosynthesis producing EPA and DHA from the precursor fatty acid ALA is not yet understood and might be successfully exploited using different approaches.

Stimulation of the *in vivo* fatty acid biosynthesis

Nutritional approach

The *in vivo* fatty acid biosynthesis comprises two main pathways (Figure GD- 1): (1) the first pathway is the synthesis of ALA to EPA which takes place in the endoplasmic reticulum, (2) the second pathway is the “Sprecher shunt” used for the production of DHA from EPA (Sprecher, 2000) located in the endoplasmic reticulum with a final β-oxidation in

the peroxisome (Burdge, 2004; Sargent et al., 2002). The key enzyme of this process is the Δ6-desaturase, which initiates the synthesis and introduces an additional double bond in ALA to form stearidonic acid (18:4n-3) (Vagner and Santigosa, 2011). The Δ6-desaturase is further involved in the desaturation of 24:5n-3 to 24:6n-3 in the "Sprecher shunt" (Figure GD- 1).

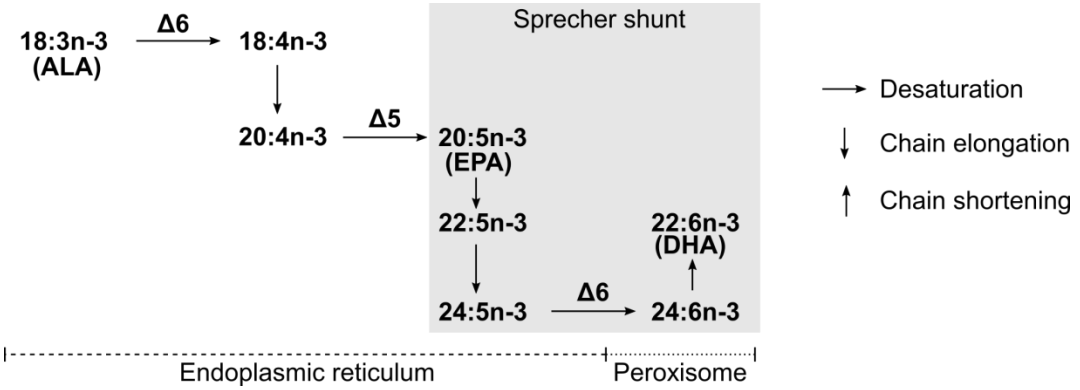

Figure GD- 1. Pathway of the hepatic *in vivo* fatty acid biosynthesis. The first pathway is the conversion of α-linolenic acid (ALA) to eicosapentaenoic acid (EPA) and the second pathway, the "Sprecher shunt", is the conversion of EPA to docosahexaenoic acid (DHA). Δ6 and Δ5 indicate desaturations by the Δ6-desaturase and the Δ5-desaturase enzymes, respectively. Graph made according to Sargent et al. (2002) and Burdge (2004).

The expression of the Δ6-desaturase, the Δ5-desaturase, the peroxisome proliferator-activated receptor α (PPARα), and some elongase genes can be upregulated when fish are fed diets with high amounts of vegetable oils (Rakhshandehroo et al., 2010; Vagner and Santigosa, 2011). This nutritional regulation is mainly caused by the increase of the substrate ALA (Cleveland et al., 2012). Furthermore, the suppression of the gene expression through EPA and DHA is relieved due to their reduced dietary amounts, thus resulting in an upregulation of the Δ6-desaturase and other genes (Leaver et al., 2008). Such nutritional regulation of the hepatic Δ6-desaturase gene was visible in rainbow trout and sea bream fed diets with reduced fish oil content (**chapters I and IV**). Additionally, a dietary upregulation of the mRNA levels of the PPARα, enoyl-CoA hydratase (ECH), and carboxyl ester lipase (CEL) genes, was visible in the nutritionally challenged sea bream (**chapter IV**). The nutritional regulation of the metabolic fatty acid synthesis pathway has evolved to satisfy the needs of the fish itself and not of us humans (Tocher, 2015). Thus, the resulting lipid quality might vary from the one favored for human nutrition. Further stimulation of the pathways can only be realized *via* other strategies, for example environmental or biochemical approaches.

Environmental approach

It is generally accepted that the main environmental factor affecting the fatty acid composition of fish is the water temperature, besides other factors like salinity or seasonal changes (Vagner and Santigosa, 2011). An adaptation of the fatty acid composition to temperature changes is necessary from a physiological point of view in order to guarantee the functionality and fluidity of membranes and other cellular functions (Sargent et al., 2002). It is accepted that the main determinant with regard to membrane fluidity is the ratio between saturated and monounsaturated fatty acids. Lower water temperatures would lead to an increase in monounsaturated in relation to saturated fatty acids (Wodtke and Cossins, 1991) and could be observed in sea bream (**chapter IV**). Furthermore, several studies have demonstrated that changes in temperature affect the fatty acid synthesis (activity and expression of genes) and amount of n-3 HUFAs in fishes (Skalli et al., 2006; Tocher et al., 2004; Vagner and Santigosa, 2011). In many fish species it seems that the fatty acid desaturation, elongation, and β-oxidation activities are increased at lower temperatures (Tocher et al., 2004; Vagner and Santigosa, 2011). Furthermore, Skalli et al. (2006) demonstrated that the amount of n-3 HUFAs were increased in the phospholipid fraction in different tissues of sea bass held at low water temperatures. In this thesis, sea bream fed low-fish oil diets and challenged with a low water temperature, had elevated EPA and DHA levels (**chapter IV**). Though changes in the fatty acid synthesis in response to temperature have been demonstrated, the effect of temperature on growth and metabolism should not be neglected and might simultaneously modulate the fatty acid composition of fish (**chapter IV**). Moreover, since temperature-mediated responses of the lipid composition are realized in order to maintain physiological functions, an increase in n-3 HUFAs might be present but maybe not sufficient to satisfy demands for human consumption.

Biochemical approach

Dietary supplementation aiming at a stimulation of the *in vivo* fatty acid synthesis might be realized *via* single bioactive substances or raw materials containing functional substances. In order to resume the principle of a substrate regulation of the fatty acid synthesis, bioactive fatty acids have been investigated. The bioactive 3-thia fatty acids, petroselinic acid (18:1n-12), conjugated linoleic acid, and stearidonic acid (18:4n-3) were supplemented to diets for salmonids and led to increased tissue n-3 HUFA levels and β-oxidation capacity through stimulation of expression of genes encoding proteins involved in the fatty acid synthesis (Berge et al., 2004; Codabaccus et al., 2011; Kennedy et al., 2006; Moya-Falcón et al., 2004; Randall et al., 2013). Besides functional lipids, bioactive secondary plant compounds may function as complements or additives in fish oil

replacement, but they cannot simply replace fish oil as lipid source in aquafeeds. The required dietary oil levels should be met by alternative plant oils, ideally providing suitable substrates (e.g. ALA) for the mode of action of bioactives. A mixture of different medicinal herbs containing a variety of polyphenolic and bioactive substances was able to increase the EPA, DHA, and total n-3 fatty acid content and improve the fatty acid utilization in Japanese flounders (Ji et al., 2007). Purple corn extracts were used to elevate the amounts of n-3 polyunsaturated fatty acids (PUFAs) in rainbow trout (Villasante et al., 2015). The use of wine lees in order to modulate the lipid metabolism and quality in zebra fish embryos (Caro et al., 2017) is of great interest for this thesis, since wine lees are rich in bioactive substances like quercetin, rutin, catechins, and *trans*-resveratrol.

The most promising bioactive investigated so far in fish nutrition might be sesamin, the main phenolic compound in sesame seeds (Pickova et al., 2011). Dietary sesamin increased the n-3 HUFA levels in juvenile barramundi fed low-fish oil diets (Alhazzaa et al., 2012). Furthermore, sesamin lead to an increase of DHA by 13% in phospholipids and by 36% in triacylglycerols in the muscle of rainbow trout and even by 32% in salmon hepatocytes (Trattner et al., 2008a, 2008b). Within this thesis, resveratrol exhibited a comparable or even superior potential to that of sesamin. A resveratrol-mediated increase in DHA by 46% (**chapter II**) and even 70% (**chapter I**) could be achieved in the whole body of rainbow trout. In sea bream, these effects were less prominent, but the EPA + DHA content in the liver was significantly increased by 26% through dietary administration of resveratrol (**chapter IV**). In accordance with the elevated n-3 fatty acid contents by resveratrol, a stimulation of the fatty acid synthesis could be observed. Resveratrol seems to interact predominantly with the Δ6-desaturase gene. The mRNA expression of the Δ6-desaturase gene was stimulated by resveratrol in sea bream (**chapter IV**). Furthermore, the abundance of the Δ6-desaturase enzyme was elevated in livers of rainbow trout fed resveratrol (**chapter I**). It is also thinkable, that resveratrol additionally increased the activity of the Δ6-desaturase enzyme, which had been observed in rat hepatocytes (Momchilova et al., 2014) but was not investigated within this thesis. Further regulatory activity of resveratrol with PPARα or genes involved in the β-oxidation of fatty acids and lipid metabolism (e.g. carnitine palmitoyl transferase 1 (CPT1), ECH, and CEL) might have been observed in several *in vitro* and *in vivo* studies with mice and rats (Cheng et al., 2009; Inoue et al., 2003; Momchilova et al., 2014; Nakata et al., 2012; Tsukamoto et al., 2010) but could neither be observed in rainbow trout nor sea bream in this thesis (**chapters I and IV**). Besides resveratrol, sesamin and genistein also seem to target the expression of genes involved in the fatty acid synthesis and lipid metabolism. Sesamin exhibited regulatory activity on lipid-related genes and transcription factors, thus stimulating the fatty acid synthesis in salmon and trout (Trattner et al. 2008a, 2008b). Genistein activates genes and transcription factors involved in the fatty acid synthesis and

metabolism, indicated by *in vitro* studies with salmon hepatocytes and *in vivo* studies with mice (Mezei et al., 2006; Schiller Vestergren et al., 2011; Takahashi et al., 2009). Moreover, investigations in human and trout hepatocytes revealed that genistein possesses the potential to elevate the Δ6-desaturase mRNA expression (Knopp, 2015; Truong, 2016). No such effects could be observed *in vivo* in rainbow trout (Cleveland and Manor, 2015) and own investigations indicated even adverse effects. Genistein had no effect on the mRNA expression of Δ6-desaturase and PPARα and downregulated the mRNA levels of CPT1a and CPT1b in rainbow trout (Billerbeck, 2016, Figure GD- 2). As a result, dietary genistein was not able to exhibit a significantly positive influence on the fatty acid composition of rainbow trout in this thesis (**chapter II**).

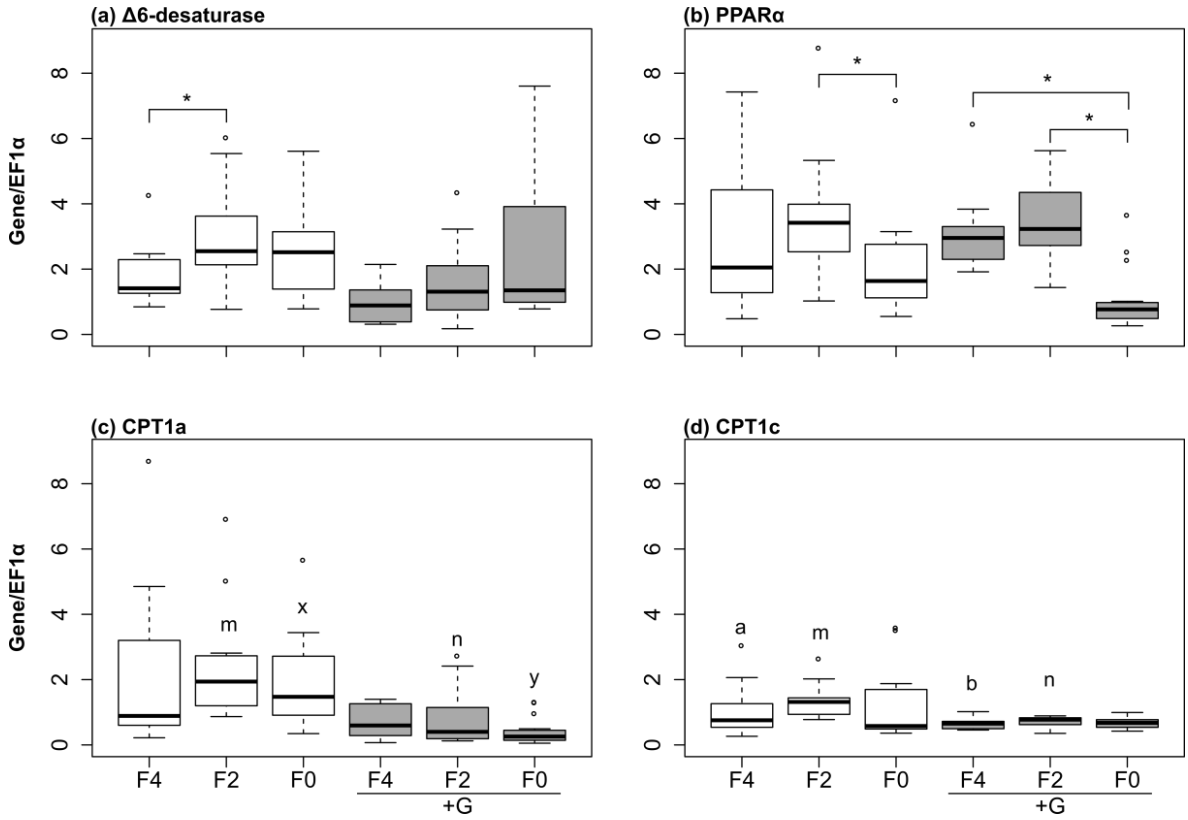

Figure GD- 2. Hepatic mRNA expression levels in rainbow trout liver following dietary treatment with varying levels of fish oil and genistein supplementation for eight weeks. **(a)** Δ6-desaturase; **(b)** PPARα; **(c)** CPT1a and **(d)** CPT1c were measured in the liver of fish using qRT-PCR and were normalized to the housekeeping gene EF1α. Feeding groups (additional groups to **chapter I**) F4, F2 and F0 were fed basal diets containing 4%, 2% and 0% dry matter fish oil, groups with +G were fed respective diets supplemented with 0.3% dry matter genistein. Boxes represent values (n = 15) between the 25 and 75 percentiles; whiskers indicate 1.5 standard deviation; the solid line indicates the median; circles represent values above and below standard deviation. Significant differences ($p < 0.05$) were analyzed using multiple contrast tests for relative effects. Tests were based on comparisons of fish oil inclusion level within one supplement group (indicated by *) or supplement type (none or +G) within one fish oil inclusion level (F4: a, b; F2: m, n; F0: x, y). Figure according to results in Billerbeck (2016).

Is there a difference between the effects of bioactives on the *in vivo* fatty acid synthesis of freshwater and marine fish?

Additionally to the difference in the response of freshwater and marine fish to the general fish oil replacement with plant oils, there can be significantly different responses to dietary bioactives, too. With regard to the aim of finding a substance that stimulates the fatty acid synthesis, conjugated linoleic acid and sesamin have exhibited markedly different responses in different fish species. Conjugated linoleic acid stimulated the *in vivo* fatty acid synthesis in juvenile salmon held in freshwater (Berge et al., 2004; Kennedy et al., 2006), but failed to show the same in the marine sea bream (Diez et al., 2007). Sesamin led to promising results in salmon and barramundi held in saltwater or brackish water, respectively (Alhazzaa et al., 2012; Trattner et al., 2008b), but had no or even a negative effect on the fatty acid synthesis of common carp (Zajic et al., 2016). Interestingly, rainbow trout were also positively affected by sesamin (Schiller Vestergren et al., 2012; Trattner et al., 2008a). Resveratrol might be a possible complement for fish oil replacement in diets for rainbow trout with regard to the fatty acid composition and synthesis, but had only marginal effects in sea bream (**chapters I, II, and IV**). A recent study reported that dietary resveratrol had no significant effect on the fillet and liver fatty acid composition of Atlantic salmon (Menoyo et al., 2018). It seems that in the case of resveratrol, there are distinctive differences in its mode of action between freshwater (rainbow trout and zebra fish) and saltwater (salmon and sea bream) fish (Caro et al., 2017; Menoyo et al., 2018 and **chapters I, II and IV**). It is assumed that in marine fish the "Sprecher Shunt" is fully functional, but the first pathway of fatty acid synthesis is disrupted (Mourente and Tocher, 1994; Tocher, 2015; Tocher et al., 2006). Since resveratrol seems to target especially the ∆6-desaturase, it might be more effective in freshwater fish where it can stimulate the complete synthesis of ALA to EPA and further to DHA. In marine fish, it would only effectively stimulate the production of DHA from EPA.

Such separation of possible modes of action might be possible for certain bioactives and feasible in marine versus freshwater fish. The boundaries between freshwater and marine lifestyles are blurred in anadromous and catadromous fish. Thus, the anadromous Atlantic salmon is an interesting subject to study the role and synthesis of HUFAs, especially during the smoltification (Tocher et al., 2003, Zheng et al., 2005). The activity of enzymes and transcription factors involved in the fatty acid synthesis peak before smoltification as adaptation to the salinity changes and decrease once the salmon has adapted to the seawater (Zheng et al., 2005.) Importantly, the genetic capacity remains the same as in the freshwater phase (Vagner and Santigosa, 2011). For example, the positive influence of sesamin on the fatty acid composition of salmon held in seawater might thus be explained by the genetic capacity and precondition determined by the preceding

freshwater phase (Trattner et al., 2008b). Nevertheless, there is considerable evidence that each bioactive has specific modes of action determined by its properties (e.g. phytoestrogen, antioxidant) (Rupasinghe et al., 2016). Besides these differences, it should not be neglected that the concentration of a bioactive determines its efficacy (Mukherjee et al., 2010; Ng et al., 2006) and can have additionally contributed to the differential responses observed in the aforementioned studies.

All in all, there are some differences in the effects of bioactives on the fatty acid synthesis between marine and freshwater fish. Some substances seem to be more efficient in marine species whereas others show greater potential for the application in freshwater fish. These differences might not solely be attributed to the bioactive and differences in the lifestyle of fish, but may underlie other factors and species specific differences.

Are effects of bioactives transferable between *in vitro* and *in vivo* and across species?

Many bioactives can interact with the lipid metabolism, lipid uptake and deposition, and energy metabolism and thus might affect the growth and nutrient composition of several animals (Ricketts et al., 2005; Rupasinghe et al., 2016). Most results were obtained in *in vitro* with human and murine hepatocytes and *in vivo* with mammals, respectively, in order to investigate weight loss and anti-obesity properties. Such effects have been demonstrated for tea catechins, coffee polyphenols, apple pectines, cocoa polyphenols, the wine polyphenol provinol, resveratrol, genistein, and many others (Aoun et al., 2010; Aprikian et al., 2003; Chang et al., 2016; Ikeda et al., 2005; Kim et al., 2004; Matsui et al., 2005; Murase et al., 2011, 2002; Yousef et al., 2004; Zang et al., 2006). Often, the transfer of results from *in vitro* to *in vivo* and across species is not fully feasible. Chlorogenic acid and alpha-tocopherol showed great potential concerning the lipid quality and metabolism *in vitro* (human and murine hepatocytes), in mice, and rats but did not affect salmon (Faizan et al., 2013; Kühn et al., 2016). In the case of genistein, the transfer of results to fish seems to depend greatly on the fish species in question. Genistein had been described to act as anti-nutritional factor impairing nutrient digestibility in salmonids (Francis et al., 2001; Kaushik et al., 1995; Olli and Krogdahl, 1995; Rumsey et al., 1994), which was in agreement with results obtained within this thesis (**chapter III**). Furthermore, genistein and other soy isoflavones led to reduced body crude lipid and growth rates in mammals and could be proven in European sea bass (Dias et al., 2005). These findings seem not to apply for rainbow trout (**chapter II**). Furthermore, results concerning the fatty acid synthesis obtained *in vitro* in salmon (Schiller Vestergren et al., 2011) and trout (Truong, 2016) hepatocytes were only partly in agreement with the effects observed *in vivo* in rainbow trout in this study.

On the other hand, resveratrol lead to similar symptoms across different animal species, though underlying mechanism may have varied. Resveratrol reduced the crude lipid content in zebra fish embryos and blunt snout bream (Caro et al., 2017; Zhang et al., 2018). In this thesis, resveratrol visibly reduced the whole body crude lipid and gross energy content of both model species (**chapters II and IV**, Table GD-1). The results allow a partial explanation of the findings in rainbow trout by indirect effects (reduced feed intake, nutrient utilization, and concurrently reduced growth, **chapter II**) and direct effects (reduced digestibility, **chapter III**). A resveratrol-mediated reduction in feed intake, feed utilization, and consequently growth had also been observed in Atlantic salmon (Menoyo, 2018). On the contrary, resveratrol did not affect the feed intake, growth, and performance of sea bream (**chapter IV** and Table GD- 1). Whether the dose administered or other mechanisms played a role in the response of growth parameters warrants further research. Nevertheless, the observed effects concerning the lipid content and quality in both species match results obtained in other animal species and *in vitro* studies to a high degree (Chang et al., 2016; Momchilova et al., 2014; Rupasinghe et al., 2016; Knopp, 2015).

Conclusively, results obtained *in vitro* can often not be transferred directly to *in vivo*, same as between different species, sometimes not even between closely related species. Even if in some cases, the results between *in vitro*, *in vivo* and different species might be similar (e.g. resveratrol), separate investigations for each species in question should be made.

Table GD- 1. Overview of effects of dietary resveratrol and genistein on different parameters of the two species rainbow trout and gilthead sea bream. The chapters and source are given in brackets. n.i. = not investigated

	Rainbow trout		Gilthead sea bream
	Resveratrol	Genistein	Resveratrol
Relative n-3 HUFA levels	Increase (I, II)	No effect (II)	Partial increase (only liver) (IV)
Fatty acid synthesis	Partial stimulation (I)	No or negative effect (Figure GD- 1)	Partial stimulation (IV)
Growth, performance	Impairment (I, II)	No effect on growth but improvement of protein utilization (II)	No effect (IV)
Whole body nutrient content	Partial impairment (II)	No effect (II)	Partial impairment (IV)
Nutrient digestibility	Partial impairment (III)	Partial impairment (III)	n.i.
Intestinal morphology	No effect (III)	No effect (III)	n.i.

Where do we go from here? Are there other alternatives?

This thesis demonstrated that two substances (resveratrol and genistein) which were hypothesized to have similar properties and exhibited similar effects in mammals and *in vitro* studies, exhibited different effects in the model freshwater species rainbow trout (Table GD-1). Whereas resveratrol had positive effects on the fatty acid composition and stimulated the fatty acid synthesis, it had negative effects on growth, performance and nutrient utilization. On the contrary, genistein had no effect on the fatty acid synthesis and composition, but slightly improved the protein utilization in rainbow trout. Consequently, the potential uses of the two substances in aquafeeds are distinctively different. Some bioactives, e.g. sesamin and resveratrol, might be potential additives for fish oil replacement in aquafeeds for freshwater fish and salmonids. The proposed potential of resveratrol needs to be further proven in carnivorous marine species and warrants further research. All in all, these bioactives have the ability to improve plant oils rich in ALA and make them comparable to fish oil with regard to tissue EPA and DHA levels. On the contrary, other bioactives, e.g. genistein, chlorogenic acid, and alpha-tocopherol, exhibited no clear practicable use for the inclusion in aquafeeds.

Complete fish oil replacements might be realized alternatively by the inclusion of novel oil sources rich in n-3 HUFAs such as krill oil, single cell oils, oils from genetically modified oilcrops, and oils from microalgae (Miller et al., 2011). Additionally, nutritional programming through specific breeding programs might be a possible way to exploit the capacity of fish to utilize vegetable oil sources more effectively (Izquierdo et al., 2015; Tocher, 2003; Turkmen et al., 2017). Each possibility aiming to improve the sustainability of aquaculture production and reduce the use of the finite resource fish oil may have advantages but also obstacles. In the case of bioactives as novel functional substances an obstacle might be that so far, no fully satisfying additive has been identified. The most promising substances so far might be resveratrol, conjugated linoleic acid, and sesamin. Though further research is especially needed in the areas of fish health, nutrient utilization, performance, and economic feasibility the successful stimulation of the *in vivo* fatty acid synthesis is forward-looking. A major advantage of nutritional regulation *via* dietary supplements would be the use of existing and readily available terrestrial oil sources instead of exploiting novel and valuable sources of EPA and DHA, which could instead be used for direct human consumption. Furthermore, the responses are rather quick and already visible within few weeks in comparison to nutritional programming involving whole production cycles. Based on the current knowledge and results obtained in this thesis, it might be of interest to investigate the application of bioactives during certain feeding periods, e.g. finishing diets or only in certain seasons (cold-adapted feed), in order to improve the quality of farmed products for human nutrition. Indispensable at

this point is, to make dose-response evaluations *in vivo* and identify harmless and most profitable concentrations of the promising bioactives.

Conclusion

This thesis provided new insight in the topic of fish oil replacement in aquafeeds for freshwater and marine fish. Functional bioactives were used as feed additives to stimulate the *in vivo* fatty acid synthesis and consequently improve the lipid quality of rainbow trout and sea bream. With regard to the stimulation of the fatty acid synthesis, resveratrol was identified as more potent additive in comparison to genistein. Thus, no added value could be identified for the use of genistein in aquafeeds. The use of resveratrol in marine fish is likely limited by the restricted and interrupted innate fatty acid synthesis. It might be suitable for the application in freshwater fish with an active *in vivo* fatty acid synthesis. For the moment, the imbalance between the stimulation of the fatty acid synthesis and simultaneous impairment of growth and performance of rainbow trout neither clearly endorses nor rejects the application of resveratrol in fish nutrition.

Since no fully satisfying bioactive for fish oil replacement has been identified so far, further research is needed to identify suitable concentrations and limits of promising bioactives in order to improve their application. This thesis contributes to a broader knowledge on the nutritional and biochemical regulation of molecular mechanisms and the use of bioactive substances in fish nutrition. The results might be further exploited to identify a possible framework for action for the balance between sustainable aquafeeds and a high lipid quality of farmed fish.

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SUMMARY

Fish and fish products contain high quality proteins and are rich in important vitamins, minerals, and essential lipids and thus contribute decisively to a healthy human nutrition. Moreover, fish and seafood are the main suppliers of omega-3 highly unsaturated fatty acids, in particular the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which play unique roles in human health and development. In aquaculture, fish is produced with aquafeeds containing fish meal and fish oil, mimicking the natural food spectrum. Fish require the nutrients and fatty acids contained in these marine resources. Furthermore, the inclusion of fish oil containing high levels of omega-3 fatty acids and EPA and DHA guarantees a good product quality for human consumption. In past years, the need for a sustainable and economic aquaculture has led to a reduction of the natural marine resources in aquafeeds. The substitution of dietary fish oil with plant oils reduced the lipid quality and amounts of EPA and DHA in aquafeeds and farmed fish. To counteract this development, it is necessary to investigate new possibilities and mechanisms and to develop innovative methods to guarantee a high lipid quality. One of the possibilities would be a stimulation of the limited endogenous fatty acid synthesis, which enables the fish to produce EPA and DHA from dietary precursor fatty acids. The fatty acid synthesis can proceed unhindered in freshwater fish whereas its function is restricted in marine fish. Besides nutritional and environmental stimulation of the fatty acid synthesis, bioactive substances own the potential to interact with the molecular mechanisms of the fatty acid synthesis. Bioactives can modify the activity and expression of involved enzymes and transcription factors, and lead to an increased production of EPA and DHA. The challenge is to identify suitable substances and to evaluate these in fish with differing natural abilities to perform the fatty acid synthesis (freshwater versus marine fish). Based on this background, the bioactive substances resveratrol and genistein were investigated for their potential to stimulate the fatty acid synthesis and to increase the amount of EPA and DHA in fish tissue. They were evaluated concerning their suitability as supplements in feeds for the freshwater species rainbow trout (*Oncorhynchus mykiss*) and the marine species gilthead sea bream (*Sparus aurata*).

Chapter I focuses on the influence of resveratrol on the endogenous fatty acid synthesis and the lipid quality of rainbow trout. Juvenile rainbow trout were fed six different diets for a period of two months. The diets had the same crude lipid content but differed in their amount of fish oil (4%, 2%, or 0% of dry matter). In addition, there was one more variant of each diet which was supplemented with resveratrol (0.3% of dry matter). The experimental evaluation revealed that resveratrol in combination with reduced dietary fish oil content positively affected the fatty acid composition of the trout. In the whole body

homogenates, the relative amounts of EPA and DHA were increased significantly, whereas the amount of the precursor omega-3 fatty acid α-linolenic acid was reduced, in comparison to control groups. Additionally, a significant increase of the absolute amounts of EPA and DHA could be ascertained in the liver. These results point to an influence of dietary resveratrol on the fatty acid synthesis of trout and were supported by means of biomolecular analyses of the liver tissue. Resveratrol was able to significantly elevate the amount of the Δ6-desaturase enzyme, which is decisively involved in the fatty acid synthesis. Additional investigations of the hepatic mRNA expression of genes involved in the β-oxidation of fatty acids admitted no further conclusions on other regulatory mechanisms of dietary resveratrol. All in all, the increased amount of the Δ6-desaturase enzyme in combination with the modified fatty acid composition leads to the conclusion, that dietary resveratrol is able to stimulate the *in vivo* fatty acid synthesis in trout.

Chapter II addresses the question, whether genistein can influence the fatty acid composition of rainbow trout the same as resveratrol. Moreover, the influence of both bioactives on the feed intake, growth parameters, and the chemical body composition was determined. Rainbow trout were fed six experimental diets for a period of two months. In this trial, the diet formulation of the preceding trial was adjusted and the diet with the intermediate fish oil content was renounced. The first diet had a fish oil content of 4% of dry matter, the second completely lacked fish oil. Both diets were fed either in this form or in their supplemented versions containing resveratrol or genistein (0.3% of dry matter). The use of resveratrol in combination with the diet lacking fish oil led to results comparable to **chapter I**, confirming its potential. Genistein had no significant influence on the fatty acid composition of the trout. Possible causes could be the dosage of genistein or its effects on regulatory mechanisms of the lipid metabolism. Both substances impaired the feed intake of the trout in a similar manner. In the case of resveratrol, this led to diminished growth and possibly caused the slightly reduced crude lipid in the fish body. In the case of genistein, the growth and the chemical body composition of the fish were not affected despite the impaired feed intake. The results indicated that a slight increase in the protein utilization could have led to this effect. Taken **chapters I and II** together, both bioactives unfolded an increased effect when they were administered in combination with low-fish oil diets. The resveratrol-mediated impairment of growth and performance indicates the need for further research.

The digestibility trial conducted in **chapter III** aimed at the analysis and identification of causes leading to the altered nutrient utilization, affected chemical body composition, and impaired growth observed in the previous trials. For a period of three weeks, rainbow trout were fed on three diets formulated according to the previously used diets. The control diet contained 0% fish oil (plant oils were the sole lipid source) and was administered in this

plain variant or was supplemented with either resveratrol or genistein (each 0.3% of dry matter). The feces of the fish were collected daily to determine the apparent digestibility of the macronutrients. Genistein reduced the digestibility of dry matter, crude lipid, and gross energy, whereas resveratrol only impaired the digestibility of gross energy. Such effects, amongst others, could have contributed to the reduced growth rates and altered chemical body composition of the rainbow trout from the preceding trials. Effects causing the reduced digestibility could not be fully determined. The histopathological investigations of the digestive tract excluded intestinal inflammations of the hindgut as cause. Based on literature data it is conceivable that resveratrol and genistein induce changes of the energy metabolism and the lipid metabolism thus influencing nutrient deposition and excretion.

Chapter IV describes the use of resveratrol as supplement in diets for gilthead sea bream in order to evaluate its potential in a marine species. Furthermore, it should be examined to which extent the impairment of growth parameters and the chemical body composition as observed in trout, applies to another fish species. Juvenile sea bream were fed on four experimental diets for the duration of two months. The diets had two different fish oil levels (6% and 2% of dry matter) and were either fed as these plain variants or were supplemented with resveratrol (0.15% of dry matter). The diets were fed to the experimental groups held either at 19 °C or 23 °C water temperature. This additional factor was included based on presumed effects of temperature on the tissue fatty acid composition and enzymes involved in the fatty acid synthesis. Since in marine fish the naturally occurring fatty acid synthesis is not fully functional, it is conceivable that a multi-factorial approach (e.g. temperature, dietary fish oil level, and feed additive) might be more effective. This trial revealed that the fatty acid compositions of the liver, the fillet, and the whole body homogenate were distinctively affected by the dietary fish oil level and the temperature. Resveratrol only caused a slight improvement of the lipid quality and significant increase of the relative amount of EPA and DHA in the livers of sea bream. This positive effect was only visible in fish held at 19 °C water temperature and fed a diet containing a fish oil level of 6%. On the contrary, a resveratrol-induced reduction of EPA and DHA was visible in fish fed held at 19 °C and fed diets containing 2% fish oil. Changes in the mRNA expression of the Δ6-desaturase gene and other genes involved in the β-oxidation of fatty acids could partially explain this bi-directional impact of resverarol. In contrast to rainbow trout, no impairment of growth and nutrient utilization and only a slight resveratrol-induced reduction of the body crude lipid content could be observed in the sea bream. Altogether, temperature as additional factor most likely exhibited effects on the lipid quality of sea bream *via* indirect mechanisms (feed intake and growth).

The initially hypothesized potential of bioactives to stimulate the *in vivo* fatty acid synthesis could only be confirmed for resveratrol. The positive effects were more pronounced in rainbow trout than in sea bream and could be explained by evolutionary adaptations of the *in vivo* fatty acid synthesis to the marine and freshwater lifestyles. Biomolecular analyses revealed a particular interaction of resveratrol with the ∆6-desaturase gene and enzyme and contributes to knowledge on the bioactive's mode of action. The efficiency of resveratrol matches the context given by other studies evaluating different bioactives in fish. An obstacle is that resveratrol can impair the growth, the nutrient digestibility, and the chemical body composition. However, this seems to be depending on the fish species, the lifestyle (marine or freshwater), and further biotic and abiotic factors. The expected stimulation of the fatty acid synthesis and improvement of the lipid quality by genistein could not be confirmed in the experimental setups carried out herein. Altogether, genistein seems to be of negligible use for fish nutrition. The studies indicated that both substances reveal bioactive activity in fish and interact with the lipid metabolism. Additionally, in the case of resveratrol, possible mechanisms of how bioactives may stimulate the fatty acid synthesis could be demonstrated. The results of this study contribute to the enlargement of the basic understanding of nutritionally regulated molecular mechanisms and indicate a possible action frame for future aquafeeds.

ZUSAMMENFASSUNG

Fisch und Fischprodukte enthalten qualitativ hochwertiges Protein und sind reich an wichtigen Vitaminen, Mineralstoffen und essentiellen Fetten und tragen maßgeblich zu einer gesunden Ernährung des Menschen bei. Zudem sind Fische und Meeresfrüchte die Hauptlieferanten der gesundheitlich förderlichen omega-3 hochungesättigten Fettsäuren, insbesondere der Eicosapentaensäure (EPA) und Docosahexaensäure (DHA). Bei der Produktion von Fischen in Aquakultur wird, entsprechend dem natürlichen Nahrungsspektrum, Fischmehl und Fischöl in den Futtermitteln eingesetzt. Einerseits benötigen Fische die darin enthaltenen Nährstoffe und Fettsäuren, andererseits wird gleichzeitig eine gute Produktqualität durch die enthaltenen omega-3 Fettsäuren EPA und DHA gewährleistet. Um die Aquakultur nachhaltiger und wirtschaftlicher zu gestalten, wurde bei der Formulierung des Fischfutters in den vergangenen Jahren immer mehr auf die natürlichen marinen Ressourcen verzichtet. Durch den Austausch der marinen Fette mit pflanzlichen Fetten in den Futtermitteln, wird die Fettqualität durch die Reduktion der EPA und DHA Mengen sowohl im Futter, wie auch im Fisch beeinträchtigt. Um dieser Entwicklung entgegenzuwirken ist es notwendig, neue Mechanismen zu erforschen und innovative Methoden zur Gewährleistung einer hohen Fettqualität zu entwickeln. Eine der Möglichkeiten ist, die limitierte endogene Fettsäuresynthese zu steigern, die es dem Fisch ermöglicht, aus Vorläuferfettsäuren EPA und DHA zu synthetisieren. Die Fettsäuresynthese kann bei Süßwasserfischen ungehindert stattfinden, wohingegen sie bei Salzwasserfischen nur bedingt funktionsfähig ist. Die Fettsäurebiosynthese kann durch gezielte Futtermittelformulierung, Umweltparameter oder bioaktive Substanzen, welche das Potential, in die Mechanismen der Fettsäuresynthese einzugreifen, besitzen, stimuliert werden. Bioaktive Substanzen können die Expression und Aktivität beteiligter Enzyme und Transkriptionsfaktoren modifizieren und letztendlich zu einer Steigerung der EPA und DHA Produktion führen. Die Herausforderung hierbei ist, geeignete Substanzen zu identifizieren und diese bei Fischen mit unterschiedlich limitierter Fettsäuresynthese zu evaluieren (Süßwasser- versus Salzwasserfische). In dieser Thesis wurden die beiden bioaktiven Substanzen Resveratrol und Genistein dahingehend untersucht, ob sie die Fettsäuresynthese stimulieren können und zu einer Erhöhung von EPA und DHA im Fisch führen. Sie wurden hinsichtlich ihrer Eignung als Additive in Futtermitteln für die Süßwasserfischart Regenbogenforelle (*Oncorhynchus mykiss*) und die Salzwasserfischart Goldbrasse (*Sparus aurata*) evaluiert.

Im Mittelpunkt von **Kapitel I** steht die Untersuchung des Einflusses von Resveratrol auf die endogene Fettsäuresynthese und die Fettsäurezusammensetzung der Regenbogenforelle. Hierzu wurden juvenile Regenbogenforellen mit sechs

unterschiedlichen Futtermitteln über einen Zeitraum von zwei Monaten gefüttert. Die Futtermittel hatten den gleichen Gesamtfettgehalt, unterschieden sich aber im Fischölgehalt (4%, 2% oder 0% der Trockenmasse). Zusätzlich gab es von jedem Futtermittel noch eine Variante, die mit Resveratrol (0.3% der Trockenmasse) versetzt wurde. Mit dieser Herangehensweise konnte festgestellt werden, dass sich Resveratrol in Kombination mit reduziertem Fischölgehalt in den Futtermitteln positiv auf die Fettsäurezusammensetzung der Forellen auswirkte. Im Ganzkörperhomogenat wurden die relativen Mengen von EPA und DHA, im Vergleich zu den Kontrollgruppen, signifikant erhöht und die Menge der Vorläuferfettsäure α-Linolensäure wurde reduziert. Zudem konnte in der Leber eine signifikante Erhöhung der absoluten Mengen von EPA und DHA festgestellt werden. Diese Ergebnisse deuten auf eine Beeinflussung des oral verabreichten Resveratrols auf die Fettsäuresynthese der Forellen hin. Mittels molekularbiologischer Analysen des Lebergewebes konnte dieses weiter vertieft werden. Resveratrol war in der Lage, die Menge des an der Fettsäuresynthese maßgeblich beteiligten Enzyms Δ6-Desaturase signifikant zu erhöhen. Die Steigerung der Enzymmenge in Kombination mit dem Veränderten Fettsäuremuster lässt auf eine gesteigerte Fettsäuresynthese bei den Forellen schließen. Zusätzliche Untersuchungen der hepatischen mRNA Expression verschiedener an der Synthese beteiligten Gene ließen keine Rückschlüsse auf weitere regulatorische Wirkmechanismen des Resveratrols zu. Insgesamt ließ sich mit diesem Versuchsteil zeigen, das Resveratrol insbesondere mit dem Enzym Δ6-Desaturase interagiert und somit das Potential besitzt, die Fettsäuresynthese und Fettqualität in Fischen zu steigern.

Kapitel II behandelt die Fragestellung, ob Genistein ein mit Resveratrol vergleichbares Potential in Hinblick auf die Beeinflussung der Fettsäurezusammensetzung von Regenbogenforellen aufweist. Zudem wurde ermittelt, welchen Einfluss beide Stoffe auf die Futteraufnahme, Wachstumsparameter und die chemische Körperzusammensetzung der Fische haben. Dazu wurden Regenbogenforellen über einen Zeitraum von zwei Monaten mit sechs Versuchsfuttermitteln gefüttert. In diesem Versuch wurden die Futtermittelformulierungen des vorangegangenen Versuchs angepasst und auf das Futter mit dem mittleren Fischölgehalt verzichtet. Somit ergab sich folgende Konstellation: Das Kontrollfuttermittel hatte einen Fischölgehalt von 4% der Trockenmasse, das andere 0%. Beide Futtermittel wurden in dieser Form oder entweder mit Resveratrol oder Genistein (0.3% der Trockenmasse) versetzt und gefüttert. Die Verwendung von Resveratrol in Kombination mit dem fischölfreien Futtermittel führte auch in diesem Versuch zu einem mit **Kapitel I** vergleichbaren Ergebnis. Es ist anzunehmen, das Resveratrol unter anderem über die Wirkmechanismen agiert hat, die in **Kapitel I** identifiziert wurden. Genistein hatte keinen signifikanten Einfluss auf die Fettsäurezusammensetzung der Forellen. Mögliche Ursachen könnten hierbei die verwendete Dosis des Genisteins oder

dessen Effekt auf regulatorische Mechanismen im Fettstoffwechsel sein. Beide Substanzen beeinträchtigten die Futteraufnahme der Forellen gleichermaßen. Im Fall von Resveratrol führte dies zu reduziertem Wachstum, welches wiederum höchstwahrscheinlich auch zu der leichten Reduktion des Rohfetts im Fischkörper führte. Im Fall von Genistein waren das Wachstum und die chemische Körperzusammensetzung der Fische trotz reduzierter Futteraufnahme nicht beeinträchtigt. Die Ergebnisse deuten darauf hin, dass unter anderem eine leichte Steigerung der Proteinverwertung der Fische hierzu geführt haben kann. Insgesamt ist in beiden Versuchen (**Kapitel I und II**) zu erkennen, dass beide Stoffe eine gesteigerte Wirkung entfalten, wenn sie in Kombination mit Futtermitteln verabreicht werden, die einen reduzierten Fischölgehalt aufweisen. Die durch Resveratrol hervorgerufenen Beeinträchtigungen deuten auf die Notwendigkeit weiterer Untersuchungen hin.

Kapitel III erläutert die Durchführung und die Ergebnisse einer Verdaulichkeitsstudie, welche zur Analyse der zuvor beobachteten Resveratrol-induzierten Beeinträchtigungen von Wachstum und chemischer Körperzusammensetzung und der Genistein-induzierten Veränderung der Nährstoffverwertung genutzt wurde. Regenbogenforellen wurden für einen Zeitraum von drei Wochen mit drei Futtermitteln aus **Kapitel II** gefüttert: das Futter mit einem Fischölgehalt von 0% der Trockenmasse wurde ohne Zusatz und mit Zusatz von Resveratrol oder Genistein (jeweils 0.3% der Trockenmasse) dargereicht. Der Kot der Tiere wurde täglich gesammelt um die scheinbare Verdaulichkeit der Makronährstoffe zu ermitteln. Genistein beeinträchtige die Verdaulichkeit der Trockenmasse, des Rohfetts und der Bruttoenergie, wohingegen Resveratrol nur die Verdaulichkeit der Bruttoenergie beeinträchtige. Solche Effekte könnten unter anderem zu den beeinflussten Wachstumsraten und veränderten chemischen Körperzusammensetzungen der Regenbogenforellen aus den vorangegangenen Versuchen beigetragen haben. Ursachen für die beeinträchtige Verdauung konnten nicht abschließend ermittelt werden. Durch histologische Untersuchungen des Verdauungstraktes konnten Entzündungen des Enddarms als Ursache ausgeschlossen werden. Auf der Basis von Literaturangaben ist es denkbar, dass Resveratrol und Genistein Veränderungen des Energiestoffwechsels und des Lipidmetabolismus verursachen und somit die Verwertung und Ausscheidung von Nährstoffen beeinflussen.

Kapitel IV beschreibt die Evaluierung von Resveratrol in der Ernährung der Goldbrasse und die Eruierung des in Regenbogenforellen sichtbaren Potentials von Resveratrol in einer marinen Art. Zudem wurde untersucht, inwieweit die in Forellen beobachteten Beeinträchtigungen des Wachstums und der chemischen Körperzusammensetzung auf eine andere Fischart zutreffen. Hierzu wurden juvenile Goldbrassen über eine Dauer von zwei Monaten mit insgesamt vier Futtermitteln gefüttert. Die Futtermittel hatten zwei

unterschiedliche Fischölgehalte, einmal 6% und einmal 2% der Trockenmasse. Zudem wurden sie in unveränderter Form verabreicht oder mit Resveratrol (0.15% der Trockenmasse) versetzt. Die Futtermittel wurden an Fischgruppen gefüttert, die entweder bei 19 °C oder 23 °C Wassertemperatur gehalten wurden. Dieser zusätzliche Aspekt begründet sich darin, dass sich die Haltungstemperatur modulatorisch auf die Fettsäurezusammensetzung und auf Enzyme der Fettsäuresynthese auswirken kann. Da in Salzwasserfischarten die Fettsäuresynthese stark beeinträchtigt ist, ist es denkbar, dass ein multifaktorieller Ansatz (Wassertemperatur, Fischölgehalt im Futtermittel und bioaktive Substanz), die Chancen einer Stimulation erhöhen. In dem Versuch konnte gezeigt werden, dass die Fettsäurezusammensetzung der Leber, des Filets und des Ganzkörperhomogenats stark von dem Fischölgehalt im Futter und der Haltungstemperatur abhängig waren. Resveratrol bewirkte nur eine sehr eingeschränkte Verbesserung der Fettqualität und führte lediglich in der Leber der Goldbrassen zu einer Erhöhung von EPA und DHA. Dieser positive Effekt war nur bei einem Fischölgehalt von 6% im Futter und nur bei 19 °C Haltungstemperatur sichtbar. Ein gegenläufiger Effekt, eine Reduktion von EPA und DHA durch Resveratrol, war sichtbar, wenn Fische Futter mit einem Fischölgehalt von 2% erhalten hatten und bei 19 °C gehalten wurden. Veränderungen in der mRNA Expression des Δ6-Desaturase-Gens und anderen Genen, die an der β-Oxidation der Fettsäuren beteiligt sind, konnten teilweise zur Erklärung dieser zweiseitigen Wirkungsweise von Resverarol herangezogen werden. Im Gegensatz zu den Forellen, konnte bei den Goldbrassen keinerlei Beeinträchtigung des Wachstums und der Nährstoffverwertung und nur eine leichte Resveratrol-induzierte Reduktion des Rohfettgehalts festgestellt werden. Insgesamt hat der zusätzliche Faktor Temperatur wahrscheinlich eher durch indirekte Mechanismen (Beeinflusste Futteraufnahme und Wachstum) Einfluss auf die Fettqualität der Goldbrassen genommen.

Mit dieser Thesis konnte durch die einzelnen Versuchsteile das eingangs angenommene Potential zur Steigerung der Fettsäuresynthese für Resveratrol bestätigt werden. Die Steigerung war in der Forelle deutlicher als in der Goldbrasse, was durch evolutive Anpassungen der Fettsäuresynthese an die Lebensweise im Salzwasser und Süßwasser zu erklären ist. Mittels molekularbiologischer Analysen konnte eine Beeinflussung des Δ6-Desaturase Gens und Enzyms, welches maßgeblich an der endogenen Fettsäuresynthese beteiligt ist, als einer der zugrundeliegenden Wirkmechanismen von Resveratrol identifiziert werden. Die beobachtete Wirkeffizienz von Resveratrol ist vergleichbar mit anderen bioaktiven Substanzen, die bereits in Fischen untersucht wurden. Als Nachteil ist zu betrachten, dass Resveratrol sich negativ auf das Wachstum, die Verdauung und die Körperzusammensetzung auswirken kann. Dies scheint aber abhängig von der Fischart, der Lebensweise (Salzwasser oder Süßwasser) und weiteren biotischen und abiotischen Faktoren zu sein. Die eingangs angenommene Stimulierung

der Fettsäuresynthese und Verbesserung der Fettqualität durch Genistein konnte im Rahmen der hier durchgeführten Versuche nicht bestätigt werden. Insgesamt scheint Genistein einen eher geringen Nutzen für den Einsatz bei der Fischölreduktion in der Ernährung von Fischen zu haben. Die durchgeführten Versuche bekräftigen, dass beide Substanzen bioaktiv wirken und mit dem Stoffwechsel der Fische, insbesondere dem Fettstoffwechsel, interagieren. Zusätzlich konnte in Hinblick auf Resveratrol eine Möglichkeit aufgezeigt werden, wie bioaktive Substanzen die endogene Fettsäuresynthese modulieren können. Somit tragen die Ergebnisse dieser Thesis zur Erweiterung des Grundverständnisses molekularer Wirkmechanismen bei und zeigen einen möglichen Handlungsrahmen für die zukünftige Fischernährung auf.

APPENDIX

Lipid extraction and fatty acid analysis

used in chapters I, II, and IV

Total lipids are extracted from homogenized diet, whole body, liver, and fillet tissue samples according to the method described by Folch et al. (1957). The amount of sample is optimized based on the total fat content to guarantee similar total fat for all measurements. The sample weights for the different tissues are: 300 mg for whole body and diet samples, 500 mg for liver and fillet tissue samples. The methylation of fatty acids and the extraction of methylated fatty acid methyl esters (FAMEs) are conducted with the help of the Folch reagent, a 2:1 mixture of chloroform and methanol. The samples are neutralized using potassium hydroxide (0.1 M) and FAMEs are isolated by the addition of the Folch reagent and subsequent centrifugation for 10 min at 2000 x *g*. The organic phase is collected and a second extraction with potassium hydroxide and the Folch reagent is performed, followed by a centrifugation (5 min at 2000 x *g*) and drying of the samples under a N_2 flux. The drying under the N_2 flux prevents the FAMEs from oxidation. At this point the sample weight represents the total content of extracted fat from the sample.

The re-dissolved FAME samples are afterwards injected into a 7820A Agilent gas chromatograph with flame ionization detector (GC-FID; Agilent Technologies, Santa Clara, CA, USA) which is equipped with an Agilent HP-88 fused silica capillary column (60 m × 250 µm × 0.2 µm, Agilent Technologies). The carrier gas used is helium at a rate of 1.2 mL/min. In order to accurately split the single FAMEs, the following temperature protocol is applied: The initial temperature is 125 °C, followed by a 26 min. lasting increase (ramp) of 8 °C/min, finally reaching 145 °C. A second 5 min. ramp follows with an increase of 2 °C/min reaching 220 °C. The recorded chromatograms (e.g. Figure A- 1) are analyzed afterwards using the EZChrom Elite software (Agilent Technologies). Prior to the measurement of the samples, a FAME standard containing all FAMEs that want to be identified is measured. The resulting chromatogram is used to identify the retention times of individual FAMEs (Figure A- 1).

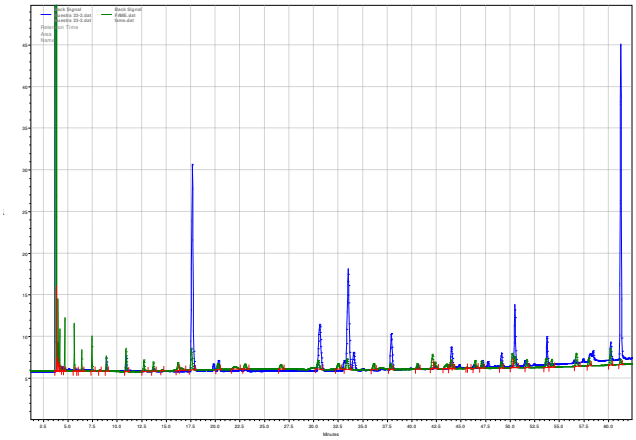

Figure A- 1. Fatty acid methyl ester (FAME) chromatogram of one sample. Each peak represents one FAME and the area is correlated to the amount of FAME in the sample. The chromatogram of the FAME standard is shown in green and blue represents the sample.

The relative abundance of each single fatty acid as percentage of all FAMEs (% FAMEs) is calculated based on the identified peak area in relation to the sum of all peak areas according to formula (A-1):

(A-1) $$fatty\ acid\ [\%\ FAMEs] = \frac{100}{\sum peak\ areas} \times individual\ peak\ area$$

To calculate the absolute amount of a single fatty acid as % of dry matter of diet (% DM) or mg/g tissue, the internal standard C13:0 methyl ester is used. The amount of fat in each sample and amount of internal standard is needed for the calculation according to formula (A-2):

(A-2) $$fatty\ acid\ [\%\ DM] = \frac{individual\ peak\ area \times internal\ standard\ [mg]}{peak\ area\ of\ internal\ standard \times fat\ [g]}$$

mRNA isolation

used in chapters I and IV

Total mRNA is extracted from the liver samples using the Innuprep RNA Mini Kit (Analytik Jena, Germany) according to the manufacturer's protocol. First, the tissue is homogenized with lysis solution and steel beads in a TissueLyser II (Qiagen, Hilden, Germany). Afterwards, the samples are filtered and washed (washing solutions containing ethanol) during multiple centrifugation steps (5 times 10.000 x g for 1 or 2 min.) in order to remove the genomic DNA and extract and purify the RNA. The resulting RNA concentration and purity are determined *via* NanoDrop measurements (NanoDrop2000c; ThermoScientific, Waltham, MA, USA) at 260, 280, and 230 nm absorbance. The solutions are diluted to a final RNA concentration of 100 ng/µl.

Quantitative real-time RT-PCR (qRT-PCR)

used in chapters I and IV

The measurement of mRNA steady state levels is realized *via* the one step quantitative real-time RT-PCR carried out in a Rotor-Gene 6000 real-time PCR cycler (Corbett/Qiagen) by using a SensiFast SYBR No-ROX One-Step Kit (Bioline, London, United Kingdom). Each PCR run includes the measurement of six internal standards in order to determine the accuracy of the run and to perform the quantitative analyses afterwards. Each mRNA sample is mixed with forward and reverse primers, the enzyme reverse transcriptase, RNase inhibitors, and SYBR-green marked nucleotides in one well of a multiwall stripe. The stripes are sealed and put in the PCR-cycler. One PCR run consist of several PCR cycles that run according to specific annealing temperatures for each set of primers. The primers and the appropriate annealing temperatures are listed in each chapter separately. The transcript expression is quantified by calculating the input copy number using a standard curve (Figure A- 2). In brief, a threshold cycle-value (CT-value), which is the intersection between an amplification curve and the threshold line, is determined for each sample. Then, the CT-values are interpolated to a standard curve (obtained from internal standards) to determine the corresponding mRNA steady state levels (concentrations).

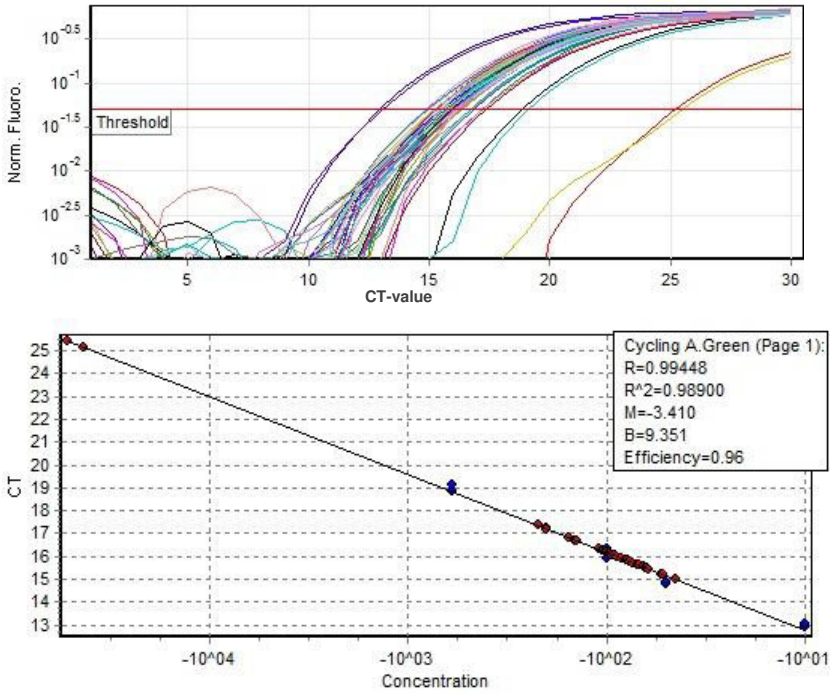

Figure A- 2. Amplification curves (upper) and the resulting standard curve (lower) for the calculation of the input copy numbers.

This is done for each target gene and the housekeeping gene. Subsequently, respective target mRNA steady state levels are normalized to the mRNA expression level of a housekeeping gene (see formula (A-3)).

(A-3) $$relative\ mRNA\ level = \frac{target\ gene\ [\frac{copies}{\mu L}]}{housekeeping\ gene\ [\frac{copies}{\mu L}]}$$

The housekeeping gene was previously identified for each fish species separately. Beta-actin (β-actin) is used for sea bream (**chapter IV**) and elongation factor 1 α (EF1α) is used for rainbow trout (**chapter I**).

Enzyme-linked immunosorbent assay (ELISA)

used in chapter I

The determination of the ∆6-desaturase protein levels is performed with a Fish Fatty Acid Desaturase 2 ELISA Kit (MBS066226, MyBiosource Inc., San Diego, CA, USA; purchased from Biozol, Eching, Germany) according to the manufacturer's instructions. The tissue samples are weighted on dry ice and lysed in phosphate buffered saline using a TissueLyser II (Qiagen, Hilden, Germany). Following centrifugation, supernatants are applied to the microELISA 96-well plate provided by the Kit. The wells are sealed with adhesive film and incubated with a HRP (horseradish peroxidase)-conjugate reagent at 37°C for 60 min. in a microplate reader. Wells are washed four times with washing solutions. The protein concentration is quantified following HRP-mediated color reaction (consecutive application of Chromogen A, B and Stop solutions) by absorbance measurements at 450 nm using a Labsystems iEMS MF multiplate reader (MTX Lab Systems, Bradenton, FL, USA purchased from Thermo Fisher Scientific, Darmstadt, Germany). The ∆6-desaturase protein concentration is calculated using a standard curve and normalized to total protein concentrations, which are evaluated *via* the Pierce bicinchoninic acid (BCA) kit (Thermo Fisher Scientific) according to the manufacturer's protocol, respectively. In brief, standards and samples are applied to a 96-well plate and working reagent is added to each well. Following incubation at 37° for 30 minutes in a microplate reader, protein concentration is measured photometrically at 540 nm and calculated against the standard curve.

Nutrient composition analysis

used in chapters II, III, and IV

The nutrient composition is determined in experimental diets, whole body, and fecal samples according to the EU guideline (EC) 152/2009 (European Union, 2009).

First, all frozen whole body and fecal samples are freeze-dried (Alpha 1-2 LDplus and Alpha 1-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until the weight is stable (between 4 and 7 days). Then, all samples are homogenized either using a cutting mill (GM 200, Retsch, Haan, Germany) for whole body samples or mortar and pestle in the case of the fecal samples and the diets.

For the determination of dry matter, the homogenized samples (2 g) are dried at 103 °C in a drying oven until the weight remains stable (4 hours).

The ash content is determined after 4 hours of incineration at 550 °C in a combustion oven (P300, Nabertherm, Lilienthal, Germany).

For the determination of crude protein (nitrogen x 6.25), the Kjeldahl method (InKjel 1225M, WD30, Behr, Düsseldorf, Germany) is used. In brief, the sample (0.5 g) is heated with sulphuric acid, which decomposes the organic substance. After distillation with sodium hydroxide, the amount of nitrogen present in the sample is determined by back titration.

The crude lipid content is quantified in the homogenized samples (2 g) following extraction with petroleum ether in a Soxleth extraction system (Soxtherm, Hydrotherm, Gerhardt, Königswinter, Germany). Samples containing plant material (e.g. the diet and fecal samples) were hydrolyzed with hydrochloric acid prior to the lipid extraction. The hydrochloric acid disrupts the plant cell walls and liberates the fat, making it accessible for the petroleum ether extractions (Eller and King, 1996).

Gross energy is measured using a bomb calorimeter (C 200, IKA, Staufen, Germany). The sample (0.5 g) is ignited in an oxygen-filled steel container and the released energy raises the temperature of the surrounding water jacket. Since there is no heat exchange between the calorimeter and surroundings, the temperature increase can be correlated to the gross energy content of the sample.

Total carbohydrates are not measured but calculated according to formula (A-4).

$$\text{Total carbohydrates} = 1000 - (\text{crude protein} + \text{crude lipid} + \text{crude ash}) \quad \text{(A-4)}$$

Analysis of TiO_2 and calculation of apparent digestibility coefficient (ADC)

used in chapter III

The amount of inert marker TiO_2 (titanium dioxide) is measured in the diet as well as the fecal samples according to DIN EN ISO 11885 (2009).

The DIN-method uses the principle of inductively coupled plasma optical emission spectrometry. In brief, ionized argon gas creates the inductively coupled plasma which produces excited atoms and ions that emit electromagnetic radiation at characteristic wavelengths. Titanium is usually determined at a wavelength of 336.121 nm. The intensity of this emission is proportional to the concentration of the element within the sample. The TiO_2 concentrations in the diet and fecal samples are used to calculate the ADC according to formula (A-5) which is based on Maynard and Loosli (1969):

(A-5) $$ADC\ (\%) = 100 \times \left[1 - \frac{(dietary\ TiO_2 \times fecal\ nutrient\ content)}{(fecal\ TiO_2 \times dietary\ nutrient\ content)}\right]$$

Histopathological analysis

used in chapter III

The hindgut samples are taken out of the 4% phosphate-buffered formalin (Roti®-Histofix 4%, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and dehydrated using a series of graded ethanol concentrations. Then, the samples are embedded in paraffin and cross-sectioned at 3 µm with a microtome. Afterwards, the cross-sections are stained with hematoxylin and eosin (HE) and fixed on microscope slides. Individual cross-sections are analyzed with a light microscope (Primo Star iLED, Carl Zeiss Microscopy GmbH, Göttingen, Germany) equipped with an 10x eyepiece and 4x/0.10, 10x/0.25, and 40x/0.65 objectives. The tissue morphology of all cross-sections is categorized following an observer-blinded assessment based on Baeverfjord and Krogdahl (1996) and Romarheim et al. (2008). The histopathological alterations and their categorization are listed in (Table A- 1):

Table A- 1. Histopathological alterations and their categorization.

Alteration type	Categories
Mucosal folds, height	Short, Medium, Tall
Submucosa, width	Thin, Wide
Lamina propria, width	Thin, Intermediate, Moderate, Wide
Enterocyte vacuolization	Low, Moderate, High
Nuclear position in enterocyte	Basal, Apical displacement
Lamina propria, infiltration	None, Slight, Moderate, Marked

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März 2009 - April 2012	**Studentische Mitarbeiterin** Zoologischen Institut, Abteilung Limnologie und Zoologisches Museum, Christian-Albrechts-Universität zu Kiel

Wissenschaftliche Publikationen

Torno, C., Staats, S., Rimbach, G., Schulz, C. (2018) Effects of resveratrol and genistein on nutrient digestibility and intestinal histopathology of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 491, 114 – 120. doi:10.1016/j.aquaculture.2018.03.020

Torno, C., Staats, S., Pascual-Teresa, S., Rimbach, G., Schulz, C. (2017) Fatty Acid Profile Is Modulated by Dietary Resveratrol in Rainbow Trout (*Oncorhynchus mykiss*). Mar. Drugs 15(8), 252. doi:10.3390/md15080252

Yohannes, E., **Grimm, C.**, Rothhaupt, K.-O., Behrman-Godel, J. (2017) The effect of parasite infection on stable isotope turnover rates of $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ in multiple tissues of Eurasian perch *Perca fluviatilis*. PLoS ONE 12(1): e0169058. doi:10.1371/journal.pone.0169058

Grimm, C., Lehmann,K., Clemmesen, C., Brendelberger, H. (2013) RNA/DNA ratio is an early responding, accurate performance parameter in growth experiments of noble crayfish *Astacus astacus*. Aquac Res, 46: 1937-1945. doi:10.1111/are.12348